STUDIES ON IDENTIFIABLE 5-HYDROXYTRYPTAMINE-CONTAINING NEURONES IN THE CENTRAL NERVOUS SYSTEMS OF SOME GASTROPOD MOLLUSCS

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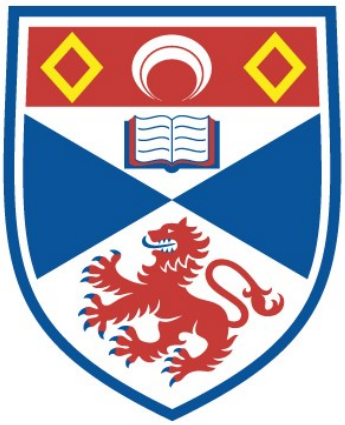

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STUDIES ON IDENTIFIABLE 5-HYDROXYTRYPTAMINE-CONTAINING NEURONES IN THE CENTRAL NERVOUS SYSTEMS OF SOME GASTROPOD MOLLUSCS.

BY

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ABSTRACT

STUDIES ON IDENTIFIABLE 5-HYDROXYTRYPTAMINE-CONTAINING NEURONES IN THE CENTRAL NERVOUS SYSTEMS OF SOME GASTROPOD MOLLUSCS.

BY

STEPHEN DOUGLAS LOGAN

Electrophysiological and neurochemical studies have been made in identifiable neurones in the central nervous systems of the gastropod molluscs Helix pomatia, Helix aspersa and Planorbis corneus.

Using a technique of intracellular injection of radioactively labelled precursors, the neurotransmitter synthesising capabilities of several neurones have been investigated. Methods have been developed to ensure that radioactive metabolites detected within the nervous system following intracellular injection are within, and confined to, the soma axons, dendrites and terminals of the injected neurones.

The results demonstrate that, with one exception some identifiable neurones possess the ability to synthesise either ^{3}H-5-hydroxytryptamine from ^{3}H-tryptophan and ^{3}H-5-hydroxytryptophan or ^{3}H-acetylcholine from ^{3}H-choline but not both transmitters from their precursors. The exception to this observation is the giant serotonin-containing neurones of Helix pomatia and Helix aspersa which can synthesise both ^{3}H-5-hydroxytryptamine and ^{3}H-acetylcholine from their precursors. Moreover this study has shown that both newly synthesised transmitters in the Helix serotonin-containing neurones are transported to the synaptic terminals of the neurone and are probably released there. Thus this neurone appears to be at variance with the concept that a neurone can utilise only one transmitter substance (Dale's Principle).

The final part of the thesis is a study of an identifiable 5-hydroxytryptamine-containing neurone in the right pedal ganglion of Planorbis corneus. This neurone has been extensively characterised by neuroanatomical, pharmacological and electrophysiological methods with a view to determining whether it makes monosynaptic connections with other neurones in the central nervous system. Although no direct connections were observed the neurone did modulate spontaneous activity in other neurones. The role of 5-hydroxytryptamine as a neuromodulator is discussed.

SUPERVISOR'S CERTIFICATE

I certify that S.D. Logan has fulfilled the conditions laid down under Ordinance General No. 12 of the University Court of St. Andrews, and is accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

DECLARATION

I declare that the work reported in this thesis is my own and has not been submitted for any other degree.

VITAE

I was educated at Annan Academy, Dumfriesshire, and the University of St. Andrews where I graduated in Physiology in 1973. The work described in this thesis was carried out between October, 1973 and March, 1977.

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Finally my thanks to my parents for my education and to Danny for her support and understanding.

CONTENTS

PART 1

ABBREVIATIONS

1. General

AAD	aromatic-L-amino acid decarboxylase
ACh	acetylcholine
AChE	acetylcholinesterase
BH_4	tetrahydrobiopterin
ChAT	choline acetyltransferase
CNS	central nervous system
DA	dopamine
DNA	deoxyribonucleic acid
L-dopa	3,4 dihydroxy-L-phenylalanine
dopa D	dopa decarboxylase
e(i)psp	excitatory (inhibitory) post synaptic potential
GABA	γ-aminobutyric acid
5-HI	5-hydroxyindole
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
5-HTPD	5-hydroxytryptophan decarboxylase
Km	Michaelis constant, affinity constant
MAO	monoamine oxidase
NA	noradrenaline
PC	phosphorylcholine
PCPA	parachlorophenylalanine
TEA	tetraethylammonium
Vmax	maximum reaction velocity

2. Identifiable neurones

ABC	anterior buccal cell (*Helix*)
GDC	giant dopamine-containing neurone (*Planorbis*)
GSC	giant serotonin-containing metacerebral neurone (*Helix*)
GSN	giant serotonin-containing pedal neurone (*Planorbis*)
GVN	giant visceral neurone (*Helix*)
MBC	medial buccal cell (*Helix*)
PBC	posterior buccal cell (*Helix*)

3. Biochemical and Physical Nomenclature

All* abbreviations for units of measurement and of physical and chemical quantities are Systeme International (SI) units.

Nomenclature for amino acids, nucleic acids and biochemical compounds are taken from : "Biochemical Nomenclature and Related Documents" (1978), Biochemical Society.

Enzyme classification is based on : "Enzyme Nomenclature : Recommendations" (1972) Elsevier Co., New York.

* Excluding the unit of radioactivity, the Curie (Ci).
In S.I., 1 Ci = 3.7×10^{10} Bequerels (Bq).

CHAPTER 1

GENERAL INTRODUCTION

1.1 Biosynthesis and metabolism of 5-hydroxytryptamine in the mammalian central nervous system.

1.1. (1) Introduction

Since 5-hydroxytryptamine (= 5-HT, = serotonin) was first reported to be a natural constituent of the mammalian central nervous system (CNS) (Twarog and Page, 1953) and to be unevenly distributed there (Amin, Crawford and Gaddum, 1954; Bogdanski, Weissbach and Udenfriend, 1957) a substantial amount of evidence has accumulated concerning its distribution, synthesis,breakdown and effects. Although the exact functional roles subserved by 5-HT in the mammalian brain remain largely conjectural, there is compelling, although in many instances circumstantial, evidence that one function of 5-HT is as a neurotransmitter (Bloom 1969; Barchas and Usdin, 1973; Green and Grahame-Smith, 1975; Marczynski, 1976). The anatomical, biochemical, electrophysiological and behavioural aspects of mammalian 5-HT systems have been extensively reviewed (Bloom 1969; Barchas and Usdin, 1973; Bloom, Hoffer Nelson, Sheu and Siggins, 1973; Costa and Sandler 1974a,b; Green and Grahame-Smith 1975, 1976; Marczynski, 1976) and as the major interest of this work is the metabolic pathways of 5-HT, only general reference will be given here with respect to the anatomical, electrophysiological and behavioural aspects of mammalian 5-HT neuronal systems.

Fluorescence histochemical and biochemical techniques have shown that 5-HT is present in the perikarya, axons, terminals and varicosities of neurones in specific tracts in the brain (Bogdanski et al.,1957; Dahlstrom and Fuxe 1964, 1965a). 5-HT containing cell bodies are mainly concentrated in the raphé nuclei and to a lesser extent in the ventromedial reticular formation of the pons and the mesencephalon.

Ascending 5-HT pathways project from these nuclei to most areas of the brain (Dahlstrom and Fuxe, 1964, 1965a; Dahlstrom, Haggendahl and Attack, 1973) and descending 5-HT bulbospinal pathways originating in nucleus raphé obscurus and nucleus raphé pallidus and some cells around the pyramidal tracts descend in lateral and anterior funiculi to spinal gray matter and are particularly prevalent in the lumbro-sacral region (Carlsson, Falck, Fuxe, and Hillarp 1964; Dahlstrom and Fuxe, 1965b).

Neuronal 5-HT is associated with synaptosomes and synaptic vesicles (Aghajanian and Bloom, 1967; Bloom, Hoffer, Siggins, Barker and Nicol, 1972; Chan-Palay, 1975, 1976; Palay and Chan-Palay, 1975; Calas, Besson, Gaughy, Alonso, Glowinski and Cheramy, 1976) and microiontophoretic application of 5-HT alters neuronal activity in almost all brain regions tested (Bloom et al., 1972; Bloom et al., 1973; Aghajanian et al., 1977). There is a high affinity uptake mechanism for 5-HT (Snyder, Shaskan and Kuhar, 1973; Iverson, 1975) and the enzymes responsible for the biosynthesis and inactivation of 5-HT can be detected in the brain (Green and Grahame-Smith 1975, and see later).

It has been suggested that 5-HT is involved in the sleep-waking cycle (Jouvet, 1973) hypothalamic control of body temperature (Myers, 1973), pain sensation, (Sicuteri, Anselmi and Del Bianco, 1973) sexual behaviour (Costa et al., 1974b). 5-HT may also play a role in several psychiatric disorders (see reviews above) including schizophrenia (Grahame-Smith, 1974) and affective disorders (Ashcroft and Glen, 1974). Several potent pharmacological agents may exert their effects via an interaction with brain 5-HT system including morphine, tricyclic antidepressants, hallucinogenic indoles, reserpine and chloropromazine (Green and Grahame-Smith, 1975).

1.1.(2) Biosynthesis

The pathways of 5-HT biosynthesis and metabolism are shown

in Figure 1.

a) Tryptophan hydroxylation

The first step in the formation of 5-HT is the hydroxylation of L-tryptophan by the enzyme tryptophan hydroxylase (L-tryptophan-5-hydroxylase. EC 1.14.16.4). First reported in brain by Gál and coworkers, (Gál, Poczik and Marshal, 1963), tryptophan hydroxylase is a mono oxygenase with three substrates; L-tryptophan. molecular oxygen and a reduced pterine cofactor (probably tetrahydrobiopterin, BH_4). The enzyme catalyses the irrerversible reaction.

$$L\text{-try} + BH_4 + O_2 \rightarrow 5\text{-HTP} + BH_2 + H_2O \qquad \text{(Gál 1974)}$$

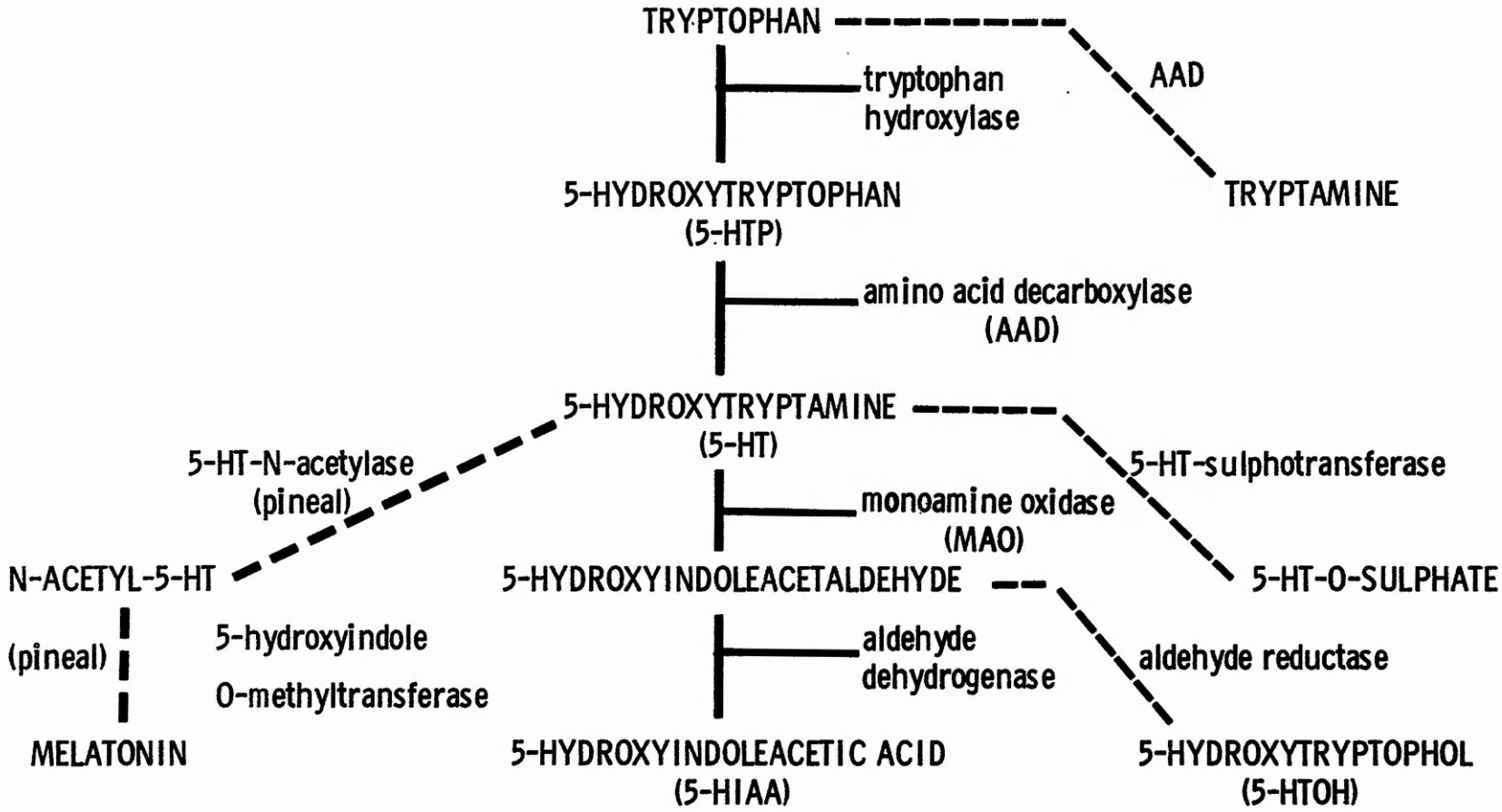
Tryptophan hydroxylase has been studied extensively *in vivo* and *in vitro* (Gál, 1974; Green and Grahame Smith, 1975) and has been partially purified from rabbit brainstem (Friedman, Kappelman and Kaufman, 1972), pig brainstem (Youdim, Hamon, and Bourgoin. 1974) and rat brainstem (Hamon, Bourgoin, Hery, Ternaux and Glowinski, 1976). The enzyme exhibits different affinity constants (Km) for its three substrates (Friedman *et al.*, 1972); the Km for tryptophan in the presence of BH_4 has a value of 5×10^{-5}M (Renson. 1973). In physiological states the enzyme is unlikely to be saturated by its substrate L-tryptophan (Friedman *et al.*, 1972; Renson, 1973; Gál, 1974) and tryptophan hydroxylation is the rate limiting step in the enzymatic synthesis of 5-HT (Grahame Smith, 1971; Carlsson, 1974; Fernstrom, Madras, Munro and Wurtman, 1974). The enzyme can be inhibited by p-chlorophenylalanine (PCPA) (Koe and Weissman, 1966) and the enzyme is probably soluble, located in the cell cytoplasm, especially in cell bodies (Lovenberg, Jequier and Sjoerdsma, 1968; Friedman *et al.*, 1976). There is also evidence for a "particulate" tryptophan hydroxylase associated with cell terminals and synaptosomes (Grahame-Smith, 1967; Ichyama, Nukamura, Nishizuka and Hayaishi, 1970; Knapp and Mandell, 1973).

Figure 1.

Metabolic pathways for the synthesis and metabolism of 5-HT. The solid line indicates the principal metabolic pathway in mammalian brain. Thin broken lines indicate less preferred pathways which may be important when the major pathway is inactivated by drugs (see Green and Grahame-Smith, 1975).

The heavy broken lines are the metabolic routes for the synthesis of melatonin in the pineal gland (see Cooper, Bloom and Roth, 1970).

METABOLIC PATHWAYS AVAILABLE FOR THE SYNTHESIS AND METABOLISM OF 5-HT



b) 5-Hydroxytryptophan decarboxylation

The conversion of 5-HTP to 5-HT requires a decarboxylation which is catalysed by the enzyme 5-hydroxytryptophan decarboxylase (5-HTPD, EC 4.1.1.28) (Bogdanski et al., 1957). 5-HTPD is probably the same enzyme that catalyses the conversion of 3,4 dihydroxy-L-phenylalanine (L-dopa) to dopamine (DA) (Kuntzman, Shore, Bogdanski and Brodie, 1961; Lovemberg, Weissbach and Udenfriend. 1962; Horney-kiewicz, 1966; Sourkes, 1966; Lloyd and Horneykiewicz, 1972). The enzyme also catalyses the decarboxylation of several other naturally occurring aromatic-L-amino acids and has been given the trivial name aromatic-L-amino acid decarboxylase (AAD) (Lovenberg et al., 1962). However, the V max for dopa is approximately ten times faster than the one for 5-HTP which is greater than that for phenylalanine, tryptophan and tyrosine (Christianson, Dairman and Udenfriend, 1972) and recent studies have shown that in brain there is an unparallel distribution of 5-HTPD and L-dopa decarboxylase (dopa D) activity (Sims and Bloom, 1971, 1973). Furthermore, 5-HTPD and dopa D have different pH and temperature optima, different activities when stimulated by pyridoxal phosphate and different intracellular locations (Sims and Bloom, 1971, 1973; Bender and Coulson, 1972; Sims 1974) However 5-HTPD and dopa D cannot be distinguished immunologically (Christian-son, et al., 1972; Hockfelt, Fuxe and Goldstein. 1973) and it has been suggested that 5-HTPD and dopa D are the same enzyme complex which has a single catalytic but separate binding sites for the two substrates (Bender and Coulson, 1972). Although AAD activity has been detected in cytoplasmic fractions of brain homogenates (Lovenberg et al, 1962; McGeer, Bagchi and McGeer, 1965) most of the activity is associated with the particulate fraction (Rodriguez de Lores Arnaiz and de Robertis,

1964; Udenfriend, 1966) and the cytoplasmic fraction activity may be an artifact of the extraction (Udenfriend, 1966). Sims and coworkers have shown that more than 50% of the 5-HTPD activity but only 24% of the dopa D activity is associated with the particulate fraction (Sims, 1974) thus supporting the evidence for two separate enzymes. At present the majority of the evidence supports the contention that there is a particulate aromatic-L amino acid decarboxylase but it is a matter for conjecture whether more than one aromatic-L-amino acid decarboxylating enzyme exists.

c) Tryptophan decarboxylation

L-tryptophan is a naturally occurring substrate for AAD (Lovenberg et al., 1962; Christianson et al., 1972) however, the decarboxylation of tryptophan to tryptamine (Figure 1) cannot be detected in normal brain tissue (Eccleston, Ashcroft, Crawford and Loose, 1966; Saavedra and Axelrod, 1973). The difference in substrate affinity exhibited by AAD for 5-HTP and tryptophan (5.4×10^{-6} M cf. 1.4×10^{-2} M, Christianson et al., 1972) and the low Km (5×10^{-5} M) Renson, 1973) for tryptophan of tryptophan hydroxylase means that the decarboxylation of tryptophan is unlikely to take place under normal conditions. Saavedra and Axelrod (1973) have shown that tryptamine, N-methyltryptamine and indole acetic acid can be detected in brain tissue after tryptophan hydroxylase and monoamine oxidase inhibition. This suggests that tryptamine synthesis from tryptophan may be insignificant in normal brain tissues.

1.1 (3) Metabolism

a) Oxidative deamination and dehydrogenation

The major degradative pathway for 5-HT in mammals is oxidative deamination followed by dehydrogenation to 5-hydroxyindole

acetic acid (Green and Grahame-Smith, 1975; (see Figure 1). The conversion of 5-HT to 5-hydroxyindoleacetaldehyde is catalysed by the enzyme monoamine oxidase (monoamine : O_2 oxidoreductase deaminating, EC 1.4.3.4, MAO) in vivo and in vitro (Neff, Young, Goridis and Bialek, 1974). The enzyme has been located in glial cells and synaptosomes (Costa and Sandler, 1972; Musacchio 1975) and appears to be associated with the outer mitochondrial membrane (Greenawalt, 1972) but there is also evidence of an extracellularly located MAO (Bieger and Hockman, 1976). There are apparently two different types of MAO in most mammalian tissues and they can be distinguished by their sensitivities to inhibitor drugs, their specificity for substrates (see Costa and Sandler 1972) and by immunological methods (Youdin, 1974). 5-HT is preferentially deaminated by type A MAO in mammalian brain (Neff et al., 1974). The product of deamination, 5 hydroxyindoleacetaldehyde is very unstable in the brain and is rapidly dehydrogenated to 5-hydroxyindole acetic acid (5-HIAA) by the enzyme aldehyde dehydrogenase (EC 1.2.1.3) (Green and Grahame-Smith, 1975).

b) Other pathways

Other metabolic pathways for 5-HT include reduction of 5-hydroxyindoleacetaldehyde to 5-hydroxytryptophol (Eccleston, Moir, Reading and Ritchie, 1966) or the sulphation of 5-HT to 5-HT-o-sulphate (Hikada, Nagatsu and Yagi 1969; Hikada and Austin, 1972). However the physiological significance of these pathways remains to be clarified (Green and Grahame-Smith, 1975).

The metabolic route of 5-HT leading to the synthesis of melatonin is also shown in Figure 1. This pathway is confined to the pineal and its immediate vicinity where an interaction between the noradrenergic

(NA) terminals from the superior cervical ganglion and the endogenous indoleamine system takes place (Klein, 1974). Noradrenaline activity through β adrenergic receptors and the adenylcyclase system promotes the synthesis of the enzyme N-acetyl-transferase (EC 2.3.1.5) which in turn catalyses the acetylation of 5-HT to N-acetyl-5-HT. The product of this reaction is then converted to N-acetyl-5-methoxy-tryptamine (or melatonin) by the enzyme hydroxyindole-o-methyl transferase (EC 2.1.1.4) (Klein, 1974).

1.1. (4) Control of 5-hydroxytryptamine synthesis

a) Tryptophan availability

Since the Km of tryptophan hydroxylase for tryptophan is much higher than the brain tryptophan concentration (Lovenberg et al., 1968) and since the enzyme is not normally saturated with its substrate (Friedman et al., 1972) small changes in brain tryptophan concentration alter the rate of 5-HT synthesis (Fernstrom and Wurtman, 1971a; Grahame-Smith, 1971). Thus the availability of the substrate tryptophan is an important step in the control of 5 HT synthesis.

Plasma tryptophan exists in a "free" (not bound to albumin) and a bound state (Knott and Curzon, 1972) and it is the "free" plasma tryptophan that is important in determining brain tryptophan, and thus brain 5-HT levels, (Tagliamonte, Biggio, Vargiu and Gessa, 1973; Curzon, 1974). Transport of tryptophan into the brain is influenced by unesterified fatty acids in plasma (Curzon, 1974), carbohydrate ingestion (Fernstrom and Wurtman, 1971b), insulin (Fernstrom and Wurtman, 1972a) and other amino acids (Fernstrom and Wurtman, 1972b).

Synaptosomes possess two concentration dependent uptake mechanisms for tryptophan which exhibit different kinetic constants (1.Km = 1.11 x 10^{-5}M; Vmax = 128 nmol.min^{-1}. (g brain)$^{-1}$; 2.Km = 3.2 x 10^{-4}M; Vmax = 621 nmol.min^{-1}. (g brain)$^{-1}$; Parfitt and Grahame-Smith, 1974).

Both uptake systems are stereospecific, temperature dependent, metabolic energy requiring and independent of changes in external Na^+ or K^+ (Green and Grahame-Smith, 1975). Other aromatic amino acids are competitive inhibitors of tryptophan uptake (Parfitt and Grahame-Smith, 1974) and synaptosomes possess an exchange diffusion mechanism for tryptophan and L-amino acids (Grahame-Smith and Parfitt, 1970; Parfitt and Grahame-Smith, 1974). Thus plasma concentrations of free tryptophan, alone, are only one determinant of 5HT synthesis.

The other two substrates for tryptophan hydroxylase, O_2 and BH_4 probably do not play a role in the regulation of 5-HT synthesis as the Km for O_2 is 2.5% (normal brain level 5%) (Friedman et al.,1972) and although tryptophan hydroxylase is sensitive to variations in O_2 concentration there is no significant reduction in the hydroxylase activity at 5.6% O_2 (Davies and Carlsson, 1973). The cofactor (BH_4) concentration is unknown in brain but the enzyme responsible for its synthesis, dihydropterine reductase, is present in large excess (Craine, Hall and Kaufman, 1972). Thus it is unlikely that either O_2 or BH_4 play a major part in the control of 5-HT synthesis.

b) Negative feedback inhibition

It has been proposed that a feedback inhibition exists at the step of tryptophan hydroxylation (Macon, Sokoloff and Glowinski, 1971; Hamon, Bourgoin, Morot-Gaudry and Glowinski, 1972; Hamon and Glowinski, 1974) and also at the 5-HTP decarboxylation step (Contractor and Jeacock, 1967). However there is considerable disagreement over the role of 5-HT in a possible feedback mechanism and several reports have suggested that if at all, only very high levels of 5-HT inhibit tryptophan hydroxylation (Jequier, Robinson, Lovenberg and Sjoerdsma, 1969; Lin, Neff, Hgai and Costa, 1969; Millard, Costa and Gál, 1972;

Renson, 1973). Consequently, there is still controversy as to whether a negative feedback inhibition controls 5-HT biosynthesis (Green and Grahame-Smith, 1975).

1.2. 5-Hydroxytryptamine in the molluscan central nervous system

1.2. (1) Introduction

There is a high concentration of 5-HT in molluscan nervous tissue (Welsh and Moorhead, 1960; Kerkut and Cottrell, 1963) which appears to be located within individual neurones (Marsden and Kerkut, 1970; Osborne and Cottrell, 1971a,b; Weinreich, McCaman and Vaughn, 1973) and associated with granular vesicles (Cottrell, 1967; Cottrell and Osborne, 1970; Schwartz, Goldman, Ambron and Goldberg, 1975; Goldman, Kim and Schwartz, 1976; Pentreath, 1976; Pentreath and Berry, 1978). Furthermore, there are selective uptake mechanisms for tryptophan, 5-HTP and 5-HT in molluscan tissue (Carpenter, Breese, Schanberg and Kopin, 1971; Pentreath and Cottrell, 1973). The 5-HT uptake mechanism is a high affinity, Na^+ dependent process which is specific for 5-HT. (Osborne, Hiripi and Neuhoff, 1974; Osborne, Schroeder and Neuhoff, 1978).

The enzymes involved in the biosynthetic pathways for 5-HT, AAD and L-tryptophan hydroxylase, are present in molluscan neurones. AAD is present it all Aplysia neurones (Weinreich, Dewhurst and McCaman, 1973) but more selectively localised in Helix neurones (Emson and Fonnum, 1974) and tryptophan hydroxylase is selectively localised in both Aplysia (Eisenstadt, Goldman, Kandel, Koike, Koester and Schwartz, 1973) and Helix (Osborne, 1973) neurones. Thus, individual neurones can synthesise 5-HT from tryptophan (Eisenstadt et al., 1973; Osborne, 1973) and 5-HTP (Cottrell and Powell, 1971; Osborne, 1972; Weinreich et al., 1973; Goldman and Schwartz, 1974).

The CNS of Aplysia, Helix and Planorbis contain degradative enzymes

which can convert 5-HT to 5-hydroxyindole acetic acid (Marsden, 1972; Osborne and Neuhoff, 1974) or conjugate 5-HT to an O-hexuronide or an aminosugar (Goldman & Schwartz, 1977).

In addition to the biochemical evidence, there is persuasive electrophysiological and pharmacological data which supports the role of 5-HT as a molluscan neurotransmitter. Early studies showed that 5-HT applied iontophoretically excites some molluscan neurones (Gerschenfeld and Stefani, 1966) and inhibits others (Glaizner, 1967; Gerschenfeld,1971). More recent studies have demonstrated at least seven pharmacologically distinct molluscan 5-HT receptors (Gerschenfeld and Paupardin-Tritsch, 1974a; Pellmar and Wilson, 1977) and that some of these receptors play a physiological role at identified central synapses in Aplysia (Gerschenfeld and Paupardin-Tritsch, 1974b). 5-HT has also been shown to play a transmitter role at peripheral synapses in Aplysia (Liebeswar, Goldman, Koester and Meyeri, 1975) and at central synapses in Helix (Cottrell, 1970, 1971, 1977; Cottrell and Macon, 1974). Thus there is compelling evidence that 5-HT functions as a neurotransmitter in the molluscan nervous system (Gerschenfeld, 1973). The majority of the data supporting a neurotransmitter role for 5-HT in molluscs has been obtained from experiments made on a pair of identifiable 5-HT containing neurones located in the cerebral ganglia of the gastropod molluscs. The following section reviews the properties of these neurones in Helix, and their homologues in other molluscs.

1.2. (2) The giant metacerebral neurones in molluscs

There is a conspicuous, bilaterally symmetrical pair of 5-HT containing neurones in the cerebral ganglia of many species of the order mollusca and these neurones appear to be homologous (Senseman & Gelperin, 1973; Weiss & Kupfermann,1974, 1976). A considerable amount of

information is available concerning the morphological, biochemical and electrophysiological properties of these neurones as well as their role in behaviour. The most comprehensive studies have been made in the pulmonate Helix by Cottrell and his coworkers (for example, see Cottrell, 1977; Osborne, 1978) and in the opisthobranch Aplysia by several groups of workers (for example, see Weiss and Kupfermann, 1976). However the metacerebral neurones of other pulmonates, Limax, (Osborne and Cottrell, 1971) and Planorbis, (Berry and Pentreath, 1976a); the opisthobranchs Tritonia (Dorsett, 1967, 1974) and Pleurobranchaea (Gillette and Davis, 1977) have also been studied.

When first identified in Helix and Limax by Cottrell and Osborne (1970) on the basis of their 5-HT-fluorescence after Falk-Hillarp treatment, the metacerebral neurones were called giant serotonin-containing cells (GSC's). Subsequent studies have not only identified homologous neurones in other species but extended the abbreviated nomenclature to describe the cells. Thus in Aplysia the neurones have been variously called cerebral 1, C1 (Weinreich et al., 1973), giant cerebral neuron, GCN (Goldman and Schwartz, 1974) and metacerebral cell, MCC (Weiss, Cohen and Kupfermann, 1975); while in Pleurobranchaea, metacerebral giant, MCG (Gillette and Davis, 1977) and in Limax and Helix, cerebral giant cell, CGC have been used. For the sake of clarity in this study it is proposed to adopt the abbreviation GSC to describe the homologous, identifiable, 5-HT-containing neurones situated in the cerebral ganglion of the gastropod molluscs. This choice has been made on the basis that although Cottrell and his coworkers were not the first to identify these neurones (Kunze, 1921), nor to examine them electrophysiologically (Kandel and Tauc 1966a,b) they were the first to

demonstrate the existence of 5-HT in the neurones (Cottrell and Osborne, 1970) and to demonstrate the monosynaptic connections between the GSC's and neurones in the buccal ganglia (Cottrell, 1970). Consequently, it is these properties that have lead to the GSC's of Helix and other species, especially Aplysia, to be studied as "models" of the mechanisms of serotonergic neurotransmission.

a) Cytoarchitecture

The GSC s of Helix Aplysia Pleurobranchaea and Planorbis are the largest neurones in the cerebral ganglia ranging in size from 100 µm in Planorbis (Berry and Pentreath, 1976a) to 300 µm in Aplysia (Weinreich et al.,1973). In Helix the cells are located on the ventral surface of the cerebral ganglia near the origin of the lip nerves and cerebro-buccal connectives (Kandel and Tauc, 1966a) whereas in Aplysia Pleurobranchaea, Tritonia and Planorbis the neurones are located in the apical region of the dorsal surface of the ganglion close to the cerebro-buccal connectives (Weinreich et al., 1973; Berry and Pentreath, 1976a; Gillette and Davis, 1977). The branching pattern of the axon of the the GSC s has been established electrophysiologically, by dye injection, $CoCl_2$ injection or by radioisotope injection followed by autoradiography (Kandel and Tauc, 1966; Dorsett, 1967; Senseman and Gelperin, 1973; Cottrell and Macon, 1974; Pentreath and Cottrell, 1974; Berry and Pentreath, 1976a; Weiss and Kupfermann, 1976; Gillette and Davis, 1977).

There are striking similarities in the gross anatomy of these neurones, for example in all the species studied the GSC's have bifurcating axons, one branch of which projects out of the external lip nerve and a second branch projects in the ipsilateral cerebro-buccal connective to the ipsilateral and contralateral buccal ganglia. This axon branch

bifurcates extensively in the buccal ganglia and small branches leave the ganglia via the three major buccal nerves, the pharyngeal nerves, and the radula nerve. In Aplysia, Helix, Pleurobranchaea and Planorbis GSC branches apparently innervate the buccal musculature via the buccal nerves and GSC stimulation modulates either directly or indirectly the activity of the buccal muscles (Pentreath, 1973; Gillette and Davis, 1975; Weiss, Cohen and Kupfermann, 1975, 1978; Berry and Pentreath, 1976a).

In Helix pomatia the branching pattern of the GSC's is more complex than in other species, (Pentreath and Cottrell, 1974) and Figure 3 (see chapter 2) shows a simplified diagram of the gross anatomy of the Helix pomatia GSC. Ipsilateral projections of the GSC are made to the ipsi and contralateral buccal ganglia and to the ipsilateral internal and external lip nerves. Contralateral projections via the cerebral commissure pass out of the cerebral ganglia via the contralateral external and internal lip nerves via the contralateral cerebro-buccal to the contralateral buccal ganglion (Pentreath and Cottrell, 1974). Electrophysiological evidence has shown that a projection via the contralateral side of the brain reaches the ipsilateral buccal ganglion (Cottrell, 1971). The most striking feature of the Helix pomatia GSC's is the presence of multiple axon branches in one nerve (Pentreath and Cottrell, 1974) (shown in Figure 3).

So far, only the GSC's Helix, Planorbis, Ariolimax and Tritonia have been shown to have projections to the contralateral cerebral ganglion (Pentreath and Cottrell, 1974; Berry and Pentreath, 1976a; Cottrell, 1977), and the GSC's of Planorbis (Berry and Pentreath, 1976a) and Ariolimax (Senseman and Gelperin, 1973) are electrically coupled.

The cell bodies of GSC's Aplysia, Helix and Tritonia contain dense-cored vesicles approximately 100 nm in diameter and 5-HT in the GSC's is, in part, associated with these vesicles as determined by histochemical and autoradiographic techniques (Cottrell and Osborne, 1970; Pentreath et al., 1973; Weinreich et al., 1973).

b) Synaptic connections

The metacerebral neurones of Helix and Aplysia make monosynaptic connections with neurones in the buccal ganglia of each species (Cottrell, 1970; Gerschenfeld and Paupardin-Tritsch, 1974b). In Helix the connections are excitatory with three identifiable buccal neurones in each buccal ganglion (Cottrell, 1970, 1971; Cottrell and Macon, 1974) and inhibitory with an unidentifiable ipsilateral buccal neurone (Cottrell, Berry and Macon, 1974). The GSC - medial buccal cell (M - cell) connection has been best studied and there is compelling evidence that this connection is monosynaptic and that 5-HT is involved in the neurotransmission process (Cottrell, 1977). There is a one to one relationship between GSC spike and M-cell epsp, even in high calcium medium; the latency of the response is constant; intracellular injection of TEA into the GSC increases the epsp amplitude; reserpine which reduces 5-HT fluorescence reduces the size of the epsp's; the GSC axons project to the close vicinity of the M-cell (Cottrell and Macon, 1974; Cottrell et al.,1974; Pentreath, 1976). These data meet several of the criteria suggested by Berry and Pentreath (1976b) for establishing monosynaptic connections.

Each Aplysia GSC makes monosynaptic connections with at least thirteen neurones in each ipsilateral buccal ganglion (Gerschenfeld and Paupardin-Tritsch, 1974b). Contralateral connections have been observed but are much weaker than the corresponding ipsilateral connections (Weiss and Kupfermann,

1976). Nine of the ipsilateral connections between the Aplysia GSC's and buccal neurones are excitatory, three inhibitory and one is an "atypical" inhibition caused by a decrease in sodium conductance (Gerschenfeld and Paupardin-Tritsch 1974b). The twelve typical connections are monosynaptic (Gerschenfeld and Paupardin-Tritsch 1974b).

Synaptic connections between Planorbis (Berry and Pentreath, 1976a) and Pleurobranchaea (Gillette and Davis, 1977) GSC s and buccal ganglion neurones are probably indirect although in some instances direct electrical connection are seen (Gillette and Davis, 1977).

c) Biosynthesis and Metabolism of 5-hydroxytryptamine in the Metacerebral Neurones

The GSC s of Aplysia, Helix, Limax, Planorbis, Pleurobranchaea and Tritonia exhibit characteristic 5-HT fluorescence after Falk-Hillarp treatment (Cottrell and Osborne, 1970; Weinreich et al.,1973; Berry and Pentreath, 1976a; Gillette and Davis, 1977). The Helix GSC s contain approximately 1 ng of 5-HT which has been detected by bioassay (Cottrell and Osborne, 1970) and micro-chemically (Osborne and Cottrell, 1971). These neurones possess an uptake mechanism for 5-HT, 5-HTP and tryptophan (Pentreath and Cottrell, 1973) and can synthesise 5-HT from exogenous tryptophan or 5-HTP (Osborne, 1972, 1973). The Aplysia GSC s contain almost 2 ng of 5-HT (D. Weinreich quoted in Goldberg, Schwartz and Sherbany, 1978) and can synthesise 5-HT from exogenous 5-HTP (Weinreich et al,1973); from endogenous tryptophan (Eisenstadt et al.,1973) and from endogenous 5-HTP (Goldman and Schwartz, 1974). AAD activity in the Aplysia GSC has an activity of 2267 pmol. $cell^{-1}.hr^{-1}$ (Weinreich et al., 1973) which is approximately four times more active than AAD in Tritonia GSC's (Weinreich et al., 1973) and forty to a hundred times more than AAD in Helix

GSC's (Emson and Fonnum, 1974; Hanley, Cottrell, Emson and Fonnum, 1974).

Newly synthesised 5-HT in Aplysia GSC's is associated with the particulate fraction after centrifugation and is transported from the cell body along the axons of the neurone (Goldman and Schwartz, 1974). in dense-core vesicles at rates of up to 3 $mm.hr^{-1}$ (Goldman, Kim and Schwartz, 1976).

1.3. Acetylcholine in the molluscan central nervous system

There is abundant electrophysiological, pharmacological and biochemical evidence which suggests that acetylcholine (ACh) functions as a molluscan neurotransmitter (McCaman and McCaman, 1976). The subject has been well reviewed recently (see Gerschenfeld, 1973; Ascher and Kehoe, 1975; Kandel, 1976, Kehoe and Marder, 1976; McCaman and McCaman, 1976) and the evidence can be summarised thus : Acetylcholine is located within ganglia and neurones (McCaman, Weinreich and Borys, 1973) along with the enzyme responsible for its synthesis from choline (choline acetyltransferase, EC 2.3.1.6., ChAT), (Giller and Schwartz, 1968; 1971; Cottrell and Powell, 1970; McCaman and Dewhurst, 1970; Emson and Fonnum, 1974). The enzyme responsible for its catabolism, acetylcholinesterase (EC 3.1.1.7., AChE), is also found in molluscan nervous tissue, (Emson and Kerkut, 1971; Giller and Schwartz, 1971; McCaman and Dewhurst, 1971; Emson and Fonnum, 1974). There are high and low affinity uptake mechanisms for choline (Schwartz, Eisenstadt and Cedar, 1975 ; Eisenstadt, Treistman and Schwartz, 1975; Osborne, 1976) and identifiable neurones can synthesise, transport and release ^{3}H-ACh after intracellular ^{3}H-choline injection (Eisenstadt et al., 1973; Koike, Eisenstadt and Schwartz, 1972; Koike, Kandel and

Schwartz, 1974; Schwartz, 1974; Eisenstadt and Schwartz, 1975; Treistman and Schwartz, 1977). Molluscan neurones possess receptors to iontophoretically applied ACh (Tauc and Gerschenfeld, 1961, 1962; Kehoe, 1972a,b). At the connections made between identifiable ACh-containing neurones and follower cells, iontophoretically applied ACh mimics the effects of stimulation of the presynaptic neurone (Kandel, Waziri and Coggeshall, 1967; Blankenship, Wachtel, and Kandel, 1971; Kehoe, 1972). Furthermore, pharmacological agents have identical effects on the iontophoretically and physiologically induced responses (Kandel et al.,1967; Blankenship et al., 1971; Kehoe, 1972c).

.4. Multiple transmitters in one neurone in molluscs

The coexistence of more than one transmitter in molluscan neurones was first suggested by Kerkut and coworkers in 1966 (Kerkut, 1969). These investigators demonstrated by chromatographic methods and the Falk-Hillarp fluorescence technique that some neurones contained both 5-HT and dopamine (Kerkut, Sedden and Walker, 1966, 1967). Subsequent studies of single neurones in Aplysia have shown the coexistence of enzymes involved in different transmitter synthesising pathways (Giller and Schwartz, 1971;Weinreich et al., 1973) and also the presence of several putative transmitters within single cells (Brownstein, Saavedra, Axelrod, Zeman and Carpenter, 1974; Cottrell, 1974;Weinreich, 1975, 1978; Saavedra, 1978).

In addition to the enzyme AAD (Emson and Fonnum, 1974) the GSC s of Helix aspersa exhibit choline acetyltransferase activity (Emson and Fonnum, 1974) and Helix pomatia GSC s are capable of in vitro synthesis of ACh from its precursors, choline and acetyl CoA (Hanley et al., 1974). Furthermore, the GSC s contain approximately 100 pg of ACh, (Hanley and

Cottrell, 1974) two thirds of which appears to be located within the cell cytoplasm (Cottrell, 1977). Electronmicroscopic studies have shown that in addition to conventional dense-core 100 nm "serotonergic" vesicles the Helix GSC's contain smaller (65 nm) clear vesicles similar to those observed in cholinergic neurones (Pentreath, 1976).

Perhaps the most interesting finding is the observation that individual post synaptic potentials, recorded in the medial buccal neurones after GSC stimulation, were reduced or abolished by hexamethonium whereas the summed depolarisation resulting from the repetitive stimulation was unaffected (Cottrell, 1976). Hexamethonium did not alter the response of the M cells to iontophoretically applied 5-HT but abolished the response to iontophoretically applied ACh (Cottrell, 1977). Thus suggesting that perhaps two transmitters are involved in the monosynaptic connection between the GSC's and the medial buccal neurones (Cottrell, 1976, 1977).

1.5. Intracellular injection of radiochemicals and plan of investigation

Intracellular injection techniques using current have been extensively used in invertebrate neurobiology to study neuronal geometry and trace the connections made by neurones (see, for example Kater and Nicholson, 1973). Substances such a Procion yellow, (Stretton & Kravitz, 1968) cobalt chloride (Pitman, Tweedle and Cohen, 1972) and radioisotopes (Pentreath, 1974) have been injected into invertebrate neurones and subsequently located by histochemistry or autoradiography.

The development by Koike, Eisenstadt and Schwartz (1972) of the pressure injecting technique has enabled relatively large amounts of radioactively-labelled substances to be introduced into neurones and enabled the subsequent metabolism and distribution of the radioactivity to be studied by biochemical techniques.

The technique has been used extensively to study the formation, fate and transport of ACh from choline (Koike et al.,1972, 1974; Eisenstadt et al.,1973; Schwartz, 1974; Eisenstadt and Schwartz, 1975; Treistman and Schwartz, 1977) and of 5-HT from tryptophan (Eisenstadt et al.,1973) and from 5-HTP (Goldman and Schwartz, 1974; Goldman et al., 1976; Goldberg et al.,1976, 1978). Intracellular pressure injection has also been used to study the formation and transport of glycoproteins in single axons (Ambron, Goldman and Schwartz, 1974, 1975) and can be applied to the study of neuronal geometry by $CoCl_2$ injection (Winlow and Kandel, 1976) or radioisotope injection followed by autoradiography (Thompson, Schwartz, and Kandel, 1976). Thus far the technique has been applied almost exclusively to Aplysia neurones reflecting, presumably, the relative ease with which larger neuronal somata can be injected.

In this study it is proposed to use intracellular injection by pressure to introduce radioactive precursors into the GSC's of Helix pomatia. These Helix pomatia neurones have been used as models of serotonergic transmission (see 1.2. (2) above) but so far the transmitter synthesising and metabolising capabilities of the GSC's have been determined by experiments made in vitro or from exogenously derived precursors. Furthermore the evidence (see 1.4. above) that 5-HT and acetylcholine may both serve as neurotransmitters within the GSC provide a unique opportunity to study a neurone which may be at variance with Dale's Principle (see Burnstock, 1977).

^{3}H-Tryptophan, ^{3}H-5-HTP and ^{3}H-choline will be injected into the GSC's and the subsequent transport and metabolism of the radioactivity studied. Other identifiable Helix pomatia neurones will also be studied particularly the medial-buccal neurone (MBC) with which the GSC makes a monosynaptic connection, and a giant neurone in the visceral ganglion (GVN,

cell 21, Kerkut, French and Walker, 1970). Both of these neurones contain the enzyme ChAT (Hanley et al., 1974) and their homologues in Helix aspersa, as well as containing ChAT, lack any AAD activity (Emson and Fonnum, 1974) and thus are presumed to be cholinergic.

Other "aminergic" neurones will also be studied including the homologous GSC s of Helix aspersa, the giant dopamine-containing neurone (GDC) of Planorbis corneus (Berry and Cottrell, 1974) and a presumed 5-HT-containing neurone in the right pedal ganglion of Planorbis corneus (see later for details). The homologous GSC s of Planorbis corneus are too small and too difficult to identify with certainty to be used in this study (Berry and Pentreath, 1976a).

The final section of the thesis will describe studies on an identified 5-HT-containing neurone in Planorbis corneus. As it seems likely that the GSC-buccal nerone connection may not be mediated by 5-HT alone (Cottrell, 1976, 1977) and that the GSC has ACh synthesising properties (Hanley, et al., 1974 and this study) it may be appropriate to discount this system as a "model" of serotonergic neurotransmission. The final section describes experiments made to determine whether the Planorbis 5-HT containing neurone makes monosynaptic connections with other neurones in the brain and if the anatomical, biochemical, electrophysiological and pharmacological properties of this neurone are similar to the other serotonin-containing neurones in molluscs.

PART 1

Chapter 2 Intracellular injection of radiochemicals : Materials and Methods.

2.1. Introduction

The majority of the experiments were made on the serotonin neurones of Helix pomatia. Analysis and location of the intraneuronal radioactivity was performed on easily identifiable structures, e.g. the cerebral ganglia, the buccal ganglia, cerebro-buccal connectives and the lip nerves. Generally the GSC somata were not dissected free from the cerebral ganglia for analysis as this increased the likelyhood of rupturing the neurone and releasing the intraneuronal contents. Thus whole tissues were extracted on the assumption that the radioactivity present was within the axons of the injected neurones (see Chapter 4,1 for further discussion). Neurones were injected with up to 30% of their volume.

2.2. (1) Dissection

Specimens of Helix pomatia and Planorbis corneus were obtained from T. Gerrard and Co., Littlehampton, Sussex. Helix aspersa were collected locally. Planorbis corneus were kept and prepared for dissection as described in detail in Chapter 5.

Helix pomatia were kept dry in large glass tanks at $4^{\circ}C$ until one week before use when they were removed to the laboratory, kept at room temperature and food was made available to them.

The snails were made active by immersing them in warm water, and the head and foot were then cut away from the shell and pinned in a wax dish. The CNS was located by cutting down the midline on the dorsal surface, exposing the buccal mass and pinning the oesophagus to the anterior of the animal. The buccal ganglia, buccal and pharyngeal nerves were carefully separated from the buccal mass leaving the nerves

Figure 2.

Photomicrograph of whole mount of Helix pomatia cerebral and buccal ganglia. The arrows indicate the giant serotonin neurones. Methylene blue has been added to the bath for greater contrast. For identification of the various structures see labelling in Figure 3.

The cerebral ganglia are viewed from their ventral aspect and the buccal ganglia are dorsal surface uppermost when pinned out in this manner.

Scale bar = 2mm

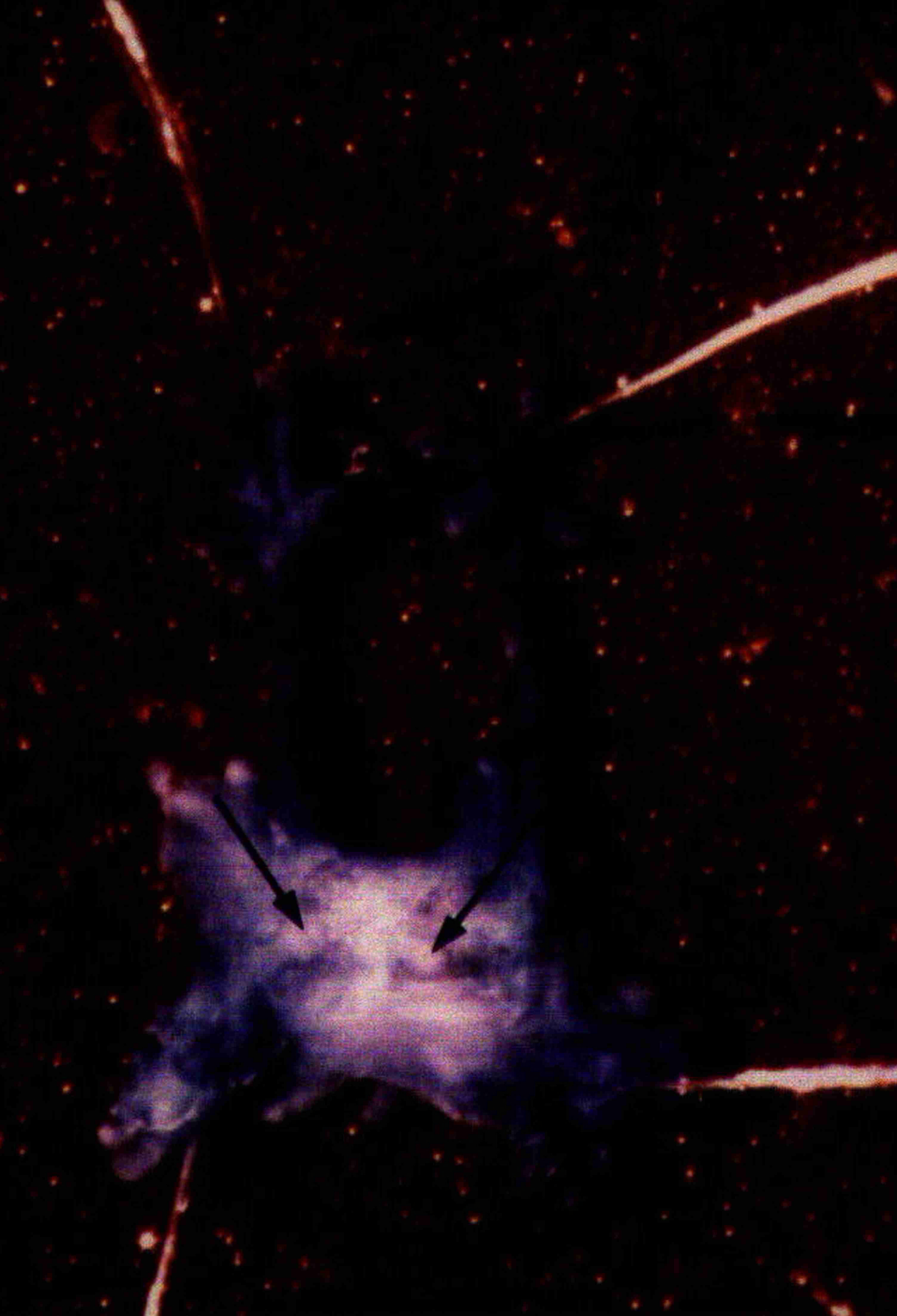
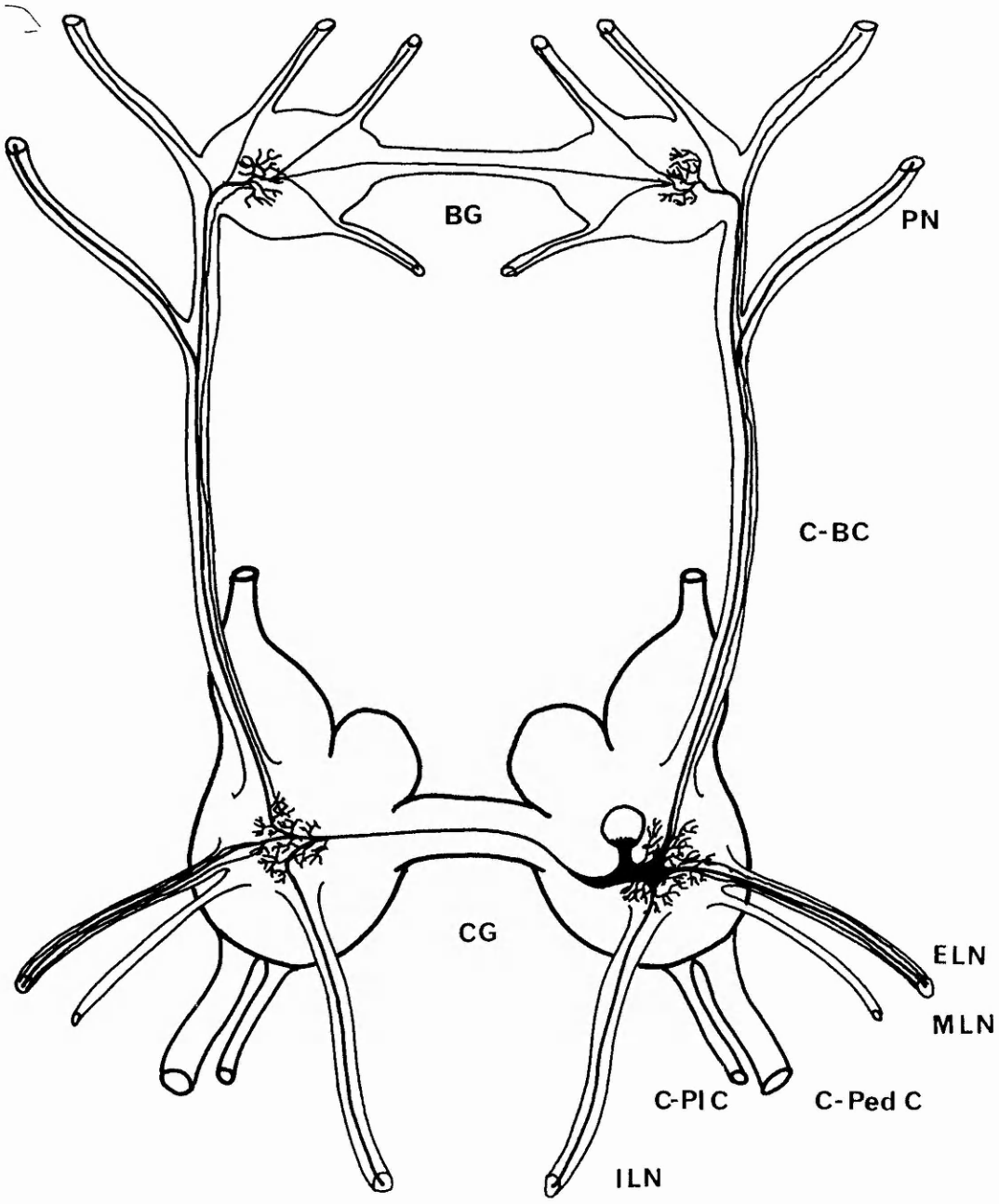


Figure 3.

Diagramatic representation of the gross morphology of one GSC from Helix pomatia (redrawn from Pentreath and Cottrell, 1974). Multiple axon branches are present in the cerebrobuccal connectives and external lip nerves. Viewed from the ventral surface.

Abbreviations : Buccal ganglia (BG); cerebral ganglia (CG); cerebrobuccal connective (C-BC); cerebropedal connective (C-Ped C); cerebropleural connective (C-PlC); external, medial and internal lip nerves (E,M and ILN); pharyngeal nerve (PN).


BG
PN
C-BC
CG
ELN
MLN
C-PlC
C-Ped C
ILN

as long as possible. Great care was taken not to damage or stress the cerebro-buccal connectives. The suboesophageal ganglia and cerebral ganglia were then freed from the rest of the animal. The lip nerves were also left long and treated carefully to minimise stress and damage. The cerebral and buccal ganglia were separated from the sub-oesophageal ganglia and pinned out as shown in Figures 2 and 3.

The connective tissue sheath surrounding the ganglia was removed by fine forceps and the underlying neurones exposed ready to be impaled with a microelectrode.

2.2. (2) Bath and Perfusion System

A special bath and perfusion system were designed in order that any radioactivity which had "leaked" into the bath was quickly diluted and removed. The design of the bath also meant that radioactivity could not diffuse from the cerebral to the buccal ganglia.

Figure 4 shows the design of the bath and indicates how the preparation was pinned out. The bath was constructed in Perspex except for the base which was hard plastic. The two grooves in the Perspex barrier were filled with a vaseline/petroleum spirit mixture (60 : 40 v/v) and after placing the two cerebro-buccal connectives into the grooves more vaseline/petroleum spirit mixture was placed over the top. This procedure did not effect the conduction velocity or the shape of the action potentials of the GSC's recorded in the connectives.

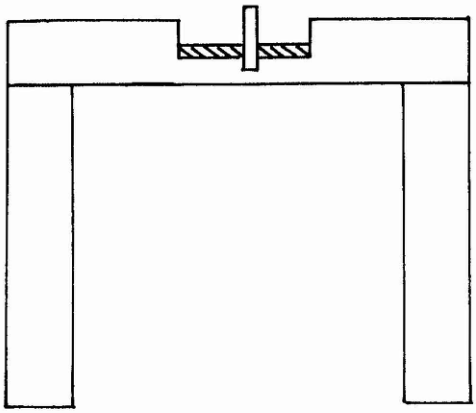
The system was checked for leakage by placing a wide tipped micro-electrode (50 μm) containing methylene blue in one chamber and watching to see if the stain diffused into the other chamber.

Two separate diffusion systems were used and to minimise inter-chamber contamination the direction of flow in each was away from the buccal ganglia (Figure 5 and see Figure 4). The ganglia were perfused

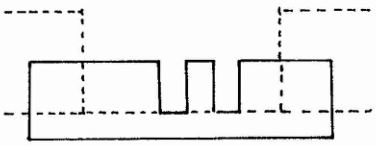
Figure 4.

Diagram of design for special bath used to isolate the buccal ganglia from site of injection (cerebral ganglia). Also indicated is the manner in which the ganglia were pinned out and the perfusion system. See text for details.

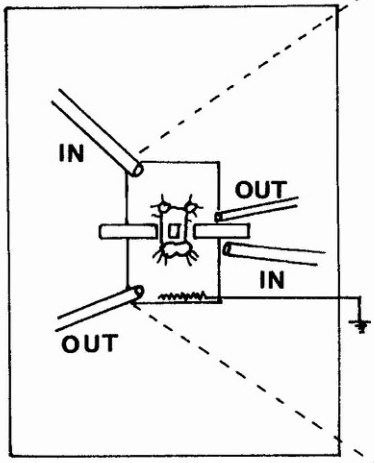
Scale bars = 1cm.



ELEVATION



PARTITION



PLAN

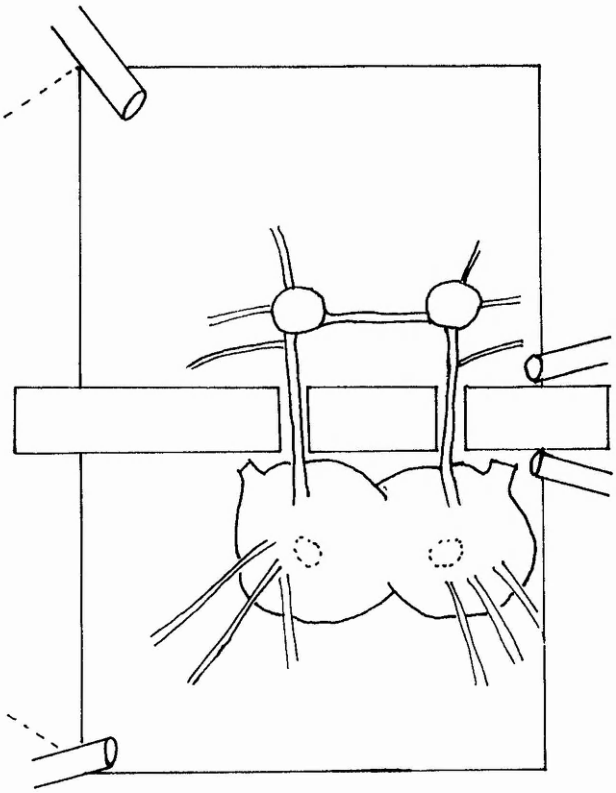
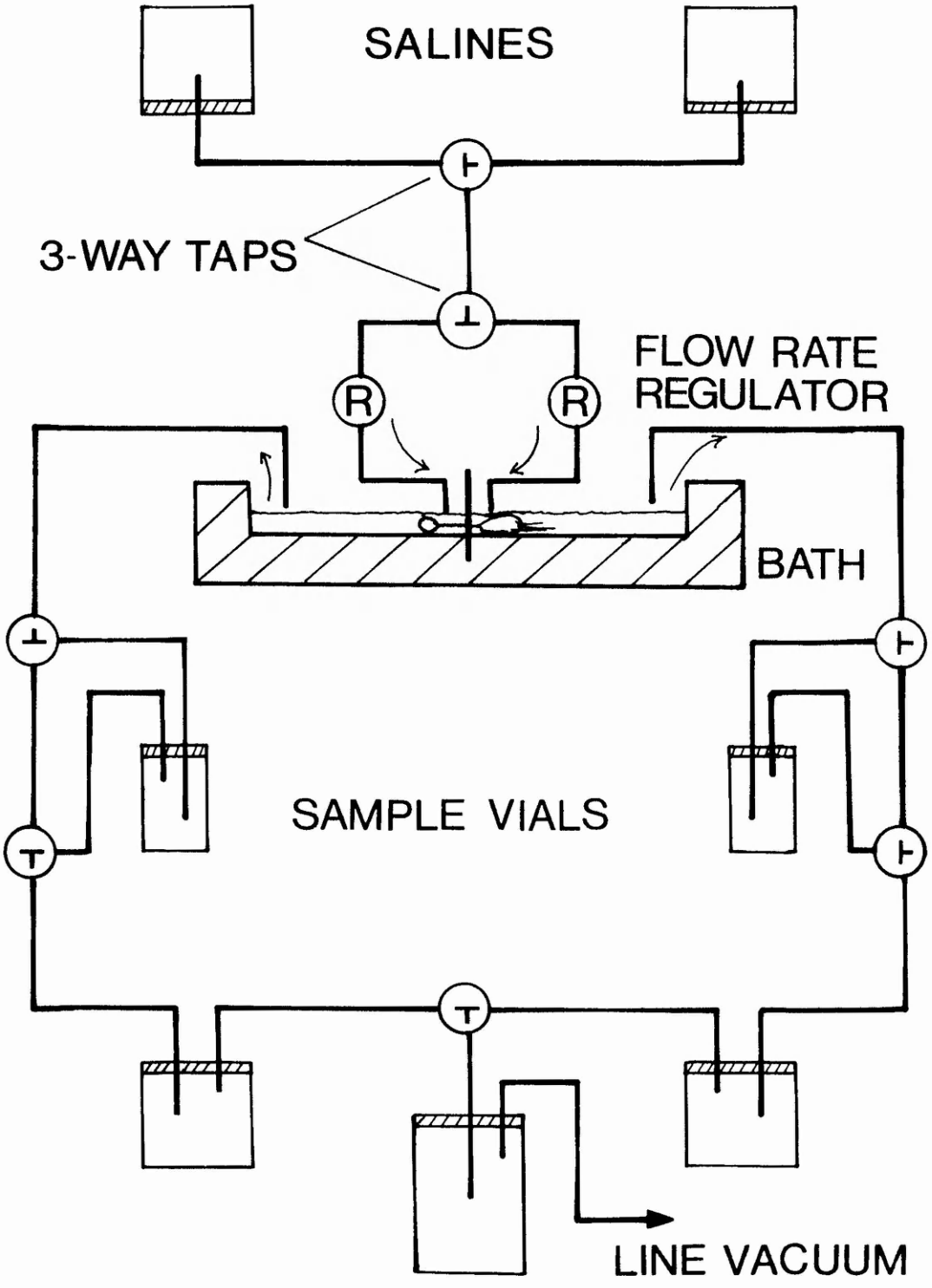


Figure 5.

Perfusion system for rapid removal and dilution of radioactivity in bath. A rapid and constant flow continuously washed radioactivity away from neuronal tissue. The system was also used to collect samples of the perfusate for analysis. The three way taps could introduce or exclude sample vials from the flow circuit thus the perfusate could be collected as required.


SALINES
3-WAY TAPS
FLOW RATE
REGULATOR
R
R
BATH
SAMPLE VIALS
LINE VACUUM

with "normal" snail solution until just before the injecting electrode was placed in the bath. At this point saline containing "cold" or unlabelled tryptophan, 5-HTP, or choline (all 50 µm) was used to perfuse the ganglia and this perfusion was continued for 30 mins after injection.

The perfusion fluid collection system is also shown in Figure 5. Three way taps were used to turn the collection vials out of the suction line. The system was designed so that the sample vials could be changed with minimum interruption to the flow. The vials were stored in order until the end of the experiment at which time the volume in each was measured and a 50 µl sample taken for counting.

In the experiments designed to identify the released radioactivity by the nerve endings in the buccal ganglia the fluid in the sample vials was reduced by evaporation *in vacuo* and subjected to electrophoresis or chromatography.

2.2. (3) Physiological solutions

The excised ganglia of *Helix* and *Planorbis* were perfused continuously with physiological solutions (*Helix*, Meng, 1960; *Planorbis*, Berry, 1972) supplemented with 1% glucose (w/v) and when required tryptophan, 5-HTP and choline (50 µm).

2.2. (4) Preparation of Radiochemicals

The following chemicals were obtained from The Radiochemical Centre, Amersham. L-(G-^{3}H)-tryptophan, 7.0 Ci.mmol^{-1}
DL-5-hydroxy (G-^{3}H) tryptophan, 4.5, 7.0, 7.1 Ci.mmol^{-1}
5-hydroxy (G-^{3}H) tryptamine creatinine sulphate, 10.7, 11.2 Ci.mmol^{-1}
(Methyl-^{3}H)-choline chloride, 10.1 Ci.mmol^{-1}

All isotopes had a radioactive concentration of 1mCi.ml^{-1} and were stored as recommended until use. Just prior to an experiment the purity of the isotope to be injected was checked by electrophoresis or

chromatography (see later for details).

0.1 to 1.0 ml of the required isotope was placed in a 25 ml pear-shaped flask (Quickfit) which was then attached to a Buchi Rotavator (Orme Scientific Instruments, Manchester) and the radiochemical was evaporated to dryness in vacuo. The dried compound was resuspended in 1-5 µl of distilled water using a Hamilton microsyringe (75N, 5 µl) and stored as a droplet on a Perspex sheet at -15°C until use.

For ^{3}H-choline injection experiments, additional purification steps were taken. The separation of ^{3}H-choline from ^{3}H-ACh by electrophoresis was approximately 1 cm (see 2.2. (7) , Table 2.1 and Figure 7), Since widening the bands of metabolites occurs progressively during electrophoresis, the ACh band and the trailing edge of the choline band could be less than 5 mm (see Figure 7). Thus some overlap of radioactive choline into the band containing standard ACh was possible. In order to minimise this possibility as narrow as band as possible was applied to the origin (see 2.2. (7) for details). Additionally, to reduce the possibility that impurities in the stock ^{3}H-choline might increase the band width or collocate with standard ACh, the stock ^{3}H-choline solution was repurified before each experiment or group of experiments using the following procedure.

0.1 - 1.0 ml stock ^{3}H-choline chloride was lyophilised, resuspended in 5 µl distilled water and subjected to electrophoresis at $10V.cm^{-1}$ (see 2.2. (7) for details). 5µl of a standard (unlabelled) choline solution (2 $\mu g.\mu l^{-1}$) was subjected to electrophoresis on a second strip in parallel with the ^{3}H-choline. Choline on the second strip was located with sublimed iodine and the corresponding region of the unstained electropherogram was eluted with 1ml 100% ethanol. 5 x 5 µl aliquots of the eluate were counted by liquid scintillation counting (see 2.2. (7)) (i) and five further 5 µl aliquots were subjected to electrophoresis (ii). The choline bands were again eluted and the radioactivity determined. A

comparison between (i) and (ii) above determined the purity of the choline at this stage(usually greater than 95%). The remainder of the ethanol ^{3}H-choline was centrifuged, lyophilised; resuspended in a droplet of distilled water (1-5 µl) and used to fill microelectrodes (see 2.2 (5)) or stored frozen until use.

2.2. (5) Preparation of electrodes

Conventional single or double barrelled glass microelectrodes containing a few strands of glass fibre were pulled so that their tips were less than 1 µm. The tip of the microelectrode was broken by touching it against tissue paper or against a Perspex rod under water until the tip size was 2-5 µm. The shorter barrel of double-barrelled electrodes was filled with either 0.5MK_2SO_4 or 2.5M KCl and used for recording and stimulating.

The longer barrel or single electrode was connected to the injection apparatus headstage (Figure 6) and the tip of the electrode was placed in distilled water. The electrode tip could be seen to fill by capillary action and the rate of filling was increased by connecting the headstage to a line vacuum.

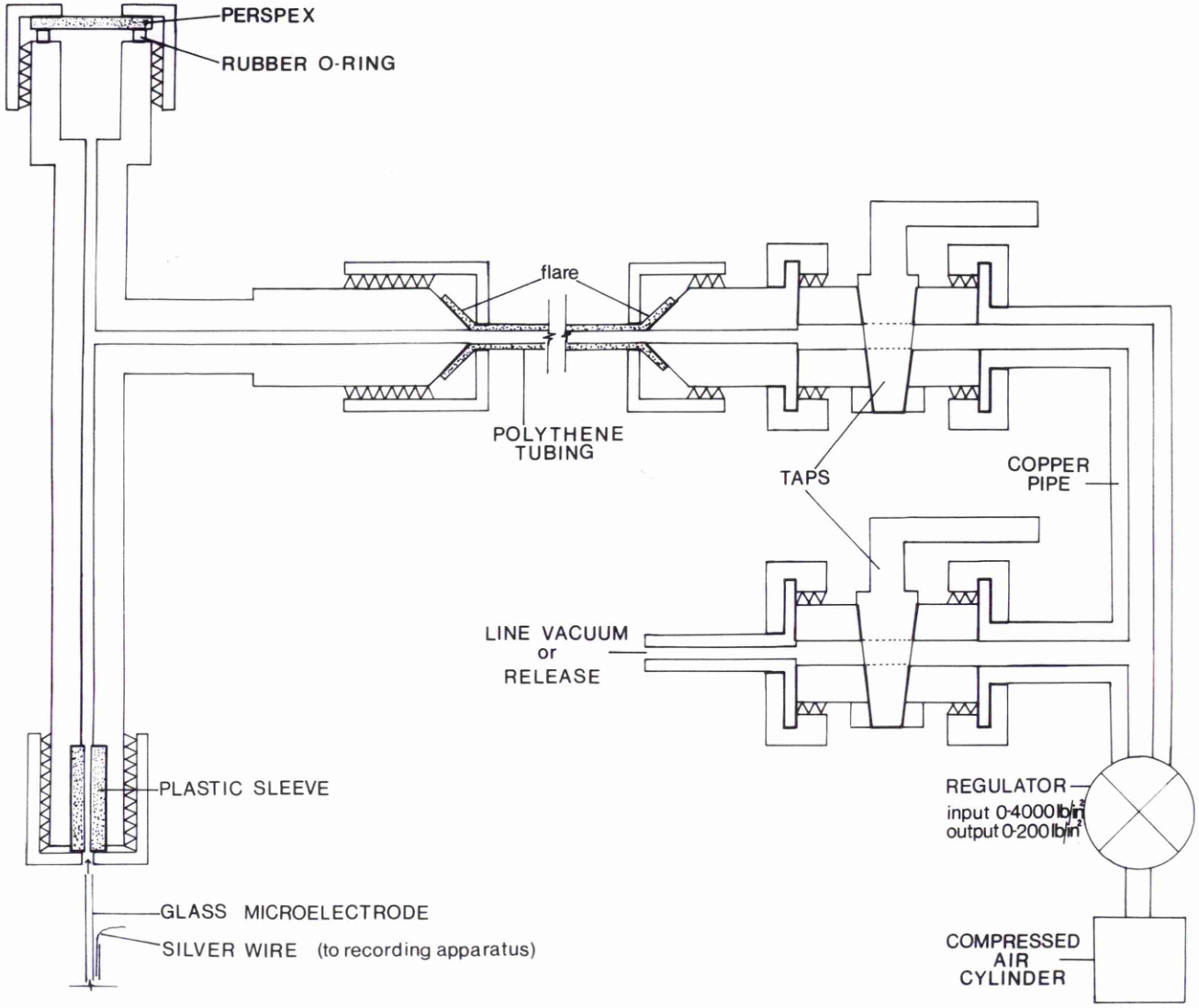
By switching from line vacuum to pressure tank the electrode could be alternately filled and emptied with distilled water. This was repeated three or four times after which the empty electrode tip was placed in the appropriate radiochemical solution and allowed to fill to a level ascertained, visually, by experience as an appropriate volume, usually 100 to 1000 pl, which corresponded to up to 30% of the GSC volume (3.2 $\pm$ 0.6 nl, n = 6 calculated as an elipsoid using the formula $\frac{4}{3}\pi a^2 b$ where a and b equal the radii of each major axis).

The electrode was then inserted into a neurone and if the neurone was undamaged as assessed by criteria such as appearance, resting potential and size or frequency of action potentials the radioactive solution was injected slowly by application of pressure (12-50 psi). The meniscus/air interface of the radioactive solution could be watched under the microscope and the

Figure 6.

Design of special microelectrode holder for pressure injection. See text for details.

(after Kater, Nicholson and Davis, 1973)


PERSPEX
RUBBER O-RING
flare
POLYTHENE TUBING
TAPS
COPPER PIPE
LINE VACUUM or RELEASE
PLASTIC SLEEVE
REGULATOR
input 0-4000 lb/in²
output 0-200 lb/in²
GLASS MICROELECTRODE
SILVER WIRE (to recording apparatus)
COMPRESSED AIR CYLINDER

pressure released when the solution had been injected. In most experiments, after the injection the electrode was removed from the neurone and a normal electrode (tip size > 1 µm) inserted in its place. Occasionally the injecting electrode was left in place but this increased the possibility of damage to the neurone. At the end of the experiment the resting potential of the neurone was recorded and those cells with resting potentials lower than -45 mV were discarded (normal, -56 ± 4 mV n = 13). No apparent differences were observed in neurones with low R.P. -45 to -52 mV compared to those in the range -52 mV to -60 mV.

2.2. (6) Pressure injecting apparatus

The microelectrode holder (Figure 6) was constructed in brass in the Gatty Marine Laboratory workshop. It is a modified version of the apparatus shown by Kater et al., (1973).

The plastic sleeve for holding the microelectrode and the polythene tubing from the taps to the headstage are the innercase covering from Uniradial 43 coaxial cable. The connecting tubing was flared and drawn out to a length of 1.5m by gentle heating and manipulation by hand.

The Perspex dish in the headstage was interchangeable with a modified disc. The modified disc had an O.B.A. screw threaded through it and Teflon coated silver wire attached to the screw and threaded through the headstage to the microelectrode where it could be inserted into the small volumes in the tips of single-barrelled microelectrodes. This technique was used only in experiments when the neurones to be injected were small or the electrodes had been "back-filled" for histological experiments.

The headstage was attached by an x-block to a brass rod held in a micromanipulator and the neurones impaled in a conventional manner. The taps and regulator (Oxygen m. 150 - 06, B.O.C) were mounted on a board and situated aside from the electrophysiological apparatus to reduce vibration. The regulator was attached to a conventional "sub-aqua" compressed air cylinder by an 'A' clamp and pressure gauge.

2.2. (7) Extraction and Analysis of Radioactivity

After the required incubation times at room temperature the ganglia and attached nerves were immersed in a 70% (v/v) ethylene glycol/physiological solution mixture and kept cold by constant application of solid CO_2 or liquid N_2 (Giller and Schwartz, 1968). The ganglia and nerves were then cut into appropriate segments using a fragment of a razor blade held in a pin vice.

Pieces of tissue were added to 10-50 µl ice cold acetone : 1 M formic acid (85 : 15) in 0.1 ml microhomogensisng tubes (Jencons Scientific Ltd.), subjected to vigorous homogenisation (20 strokes with a glass pestle and left on ice for 20 minutes. After the addition of internal standard (5 µl, 2 µg μl^{-1}), the tubes were centrifuged in a MSE centrifuge (500 r.p.m., 30 mins). The supernatants were then applied to the centreline of a strip of Watmann 3 MM paper (34 x 3 cm) and electrophoresed at 10 Vcm^{-1} for 1 h (choline) or 2 h (tryptophan) in a 1.5 M acetic acid/0.75 M formic acid buffer (pH 2.5) (Potter and Murphy, 1967). After ^{3}H-choline injection the pellet was further extracted with chloroform : methanol (1 : 1) to measure ^{3}H-choline incorporation into lipid. ^{3}H-tryptophan and other indoles were also separated by ascending paper chromatography in butanol : acetic acid : water (12 : 3 : 5). The Rf values are shown in table 2.1.

Figure 7 demonstrates diagramatically the extent of separation of the different metabolites. Choline and its derivatives were located with sublimed iodine and the indoles with Ehrlich's Reagent (10% p-dimethyl amino benzaldehyde in conc. HCl (1 Vol) + acetone (4 Vols) (Smith, 1969). After location, the strips were warmed to remove iodine or allowed to dry (indoles), cut into 5 mm strips, placed in scintillation

Figure 7.

Diagramatic representation of separation of metabolites by either ascending chromatography in butanol : acetic acid : water, 5 : 3 : 2 (solvent 1) or by electrophoresis in an acetic acid : formic acid buffer, pH 2.5 at $10 Vcm^{-1}$. 0 = origin or starting point.

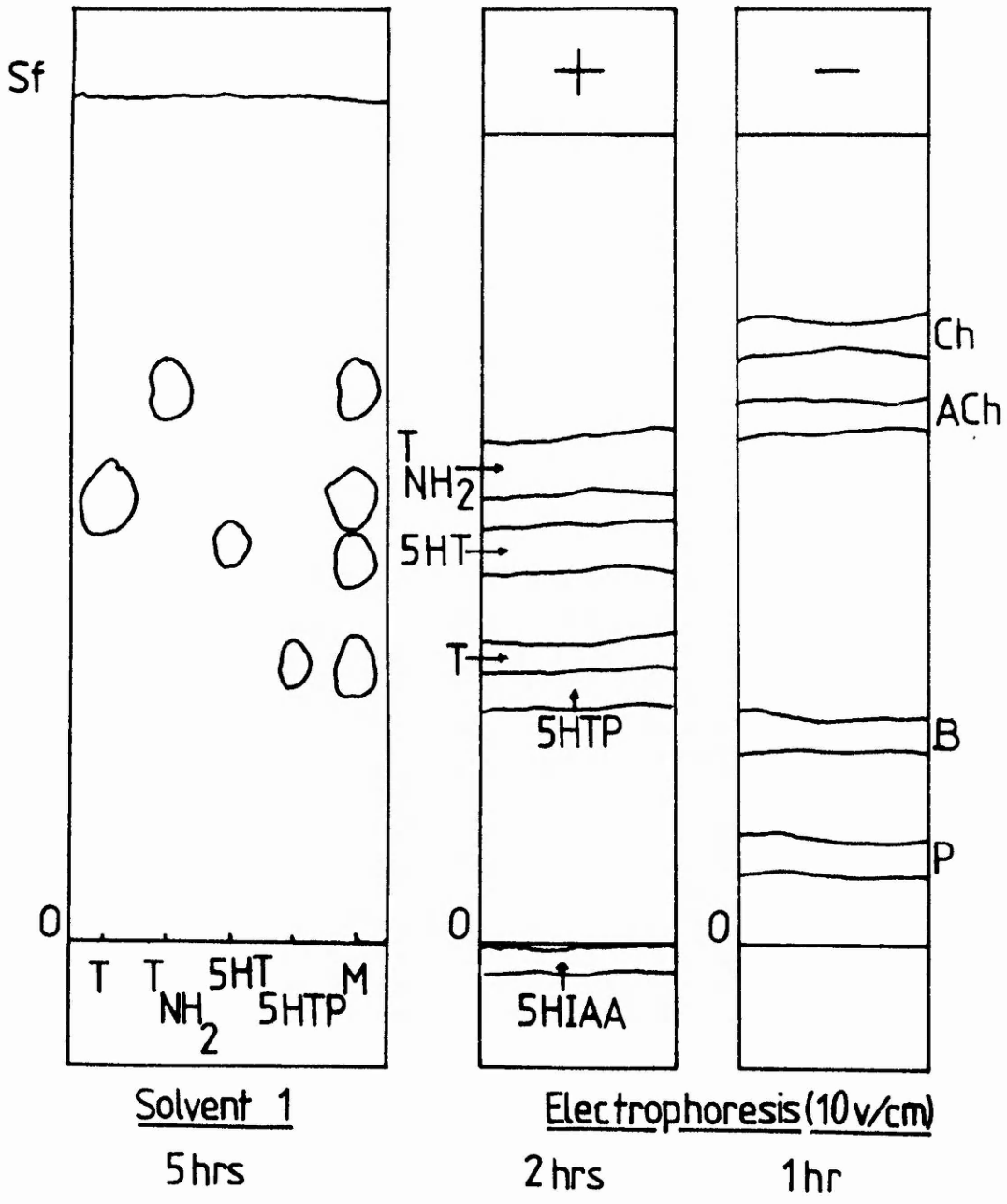
Key 1. Tryptophan and metabolites : tryptophan (T); tryptamine ($^{T}NH_2$); 5-hydroxytryptophan (5-HTP); 5-hydroxytryptamine (5-HT); 5-hydroxyindoleacetic acid (5-HIAA); mixture (m).

2. Choline and metabolites : choline (C); acetylcholine (ACh); betaine (B); phosphorylcholine (P).

Table 2.1 (below) gives calculated Rf values and nobilities.

Table 2.1. Separation of metabolites

	Rf (x 100)	Mobility ($cm.hr^{-1}$)
Tryptophan	53	2.3
Tryptamine	67	3.8
5-HT	47	3.2
5-HTP	33	2.1
5-HIAA	79	-0.3
5-HI	-	1.1
Choline	-	-9.8
ACh	-	-8.6
Betaine	-	-3.3
Phosphorylcholine	-	-1.4


Sf
0
T
T NH2
5HT
M
5HTP
+
T NH2
5HT
T
5HTP
0
5HIAA
−
Ch
ACh
B
P
0
Solvent 1
5 hrs
Electrophoresis (10 v/cm)
2 hrs
1 hr

vials (Pathlab Supplies Ltd., Kent) containing 0.6 ml 0.5 M HCl and left overnight to elute any radioactivity.

10 ml PCS solubilizer (Amersham Searle) was then added to the vials which were cooled and stored in the dark for 24 hours before counting in a Hewlett Packard Tricarb Liquid Scintillation Counter with an efficiency of 26.5%. Under these conditions of counting 1 p mol of radioisotope was equivalent to the following counts per minute.(cpm).

	$Ci.mmol^{-1}$	cpm
^{3}H-tryptophan	7.0	4118
^{3}H-5-HTP	7.0	4118
	7.1	4177
	4.5	2647
^{3}H-5-HTP	10.7	6295
	11.2	6589
^{3}H-choline	10.1	5942

For direct counting of radioactivity in tissue, samples of the CNS were placed in scintillation vials and 0.1 to 1.0 ml NCS Tissue Solubilizer (Amersham Searle) was added. The vials were left at room temperature until the tissue was completely solubilised after which 10 ml PCS scintillant was added, the vials stored and the radioactivity counted as described above.

Control experiments were made to determine the loss of radioactivity in the extraction and identification procedures. Known amounts of radioisotopes were added to 10 mg samples of snail nervous tissue which was immediately homogenised, centrifuged and the supernatant subjected to electrophoresis followed by elution of the radioactivity from the electropherogram. Losses were determined at the end of the (i) extraction, (ii) electrophoresis and (iii) elution.

Significant differences from control values were determined using

the Students 't' test.

For all four compounds used (^{3}H-tryptophan, ^{3}H-5HTP, ^{3}H-5HT and ^{3}H-choline) there were no significant differences between control and experimental values for stages (ii) and (iii) above. The losses which occurred during the extraction of each radiochemical from the snail nervous tissue varied from substance to substance. ^{3}H-5HT experimental values were not significantly different from control values ($p > 0.1$) but for ^{3}H-tryptophan ($p < 0.01$), ^{3}H-5HTP ($p < 0.1$) and ^{3}H-choline ($p < 0.1$) significant differences occurred. The following factors were calculated so that future results could be corrected for the losses, ^{3}H-tryptophan, x 1.25; ^{3}H-5HTP, x 1.15; ^{3}H-choline, x 1.18 :

2.2. (8) Electrophysiological Apparatus

Conventional recording and stimulating techniques were used and extensive details of the apparatus is given in Chapter 5.2.

2.2. (9) "Single" Neurone Experiments

In order to reduce the possibility that radioisotopes might leak out of the GSC and contaminate the immediate tissue, experiments were performed in which the GSC soma was freed from the surrounding tissue, held and injected.

Fine pins, and razor blade fragments held in pin vices were used to gently tear away the tissue and neurones surrounding the GSC. Once freed, the apparatus shown in Figure 8 was used to "lasso" the neurone by manipulating the loop of fine tunsten wire (12.5 or 25 µm) over the soma of the neurone and gently tightening the wire by means of the adjusting screw. The loop was not tightened sufficiently to ligature the axon completely and electrodes could then be inserted into the neurone in a conventional manner.

The success rate of this method was very low, initially only 1 in 3 neurones could be dissected free without damage and only 25% of these could be successfully impaled and injected. Although, from 26 animals (i.e. 52 GSC's) 15 injections were considered to be successful (c.f. > 90% for intact preparations).

2.2. (10) Chemicals

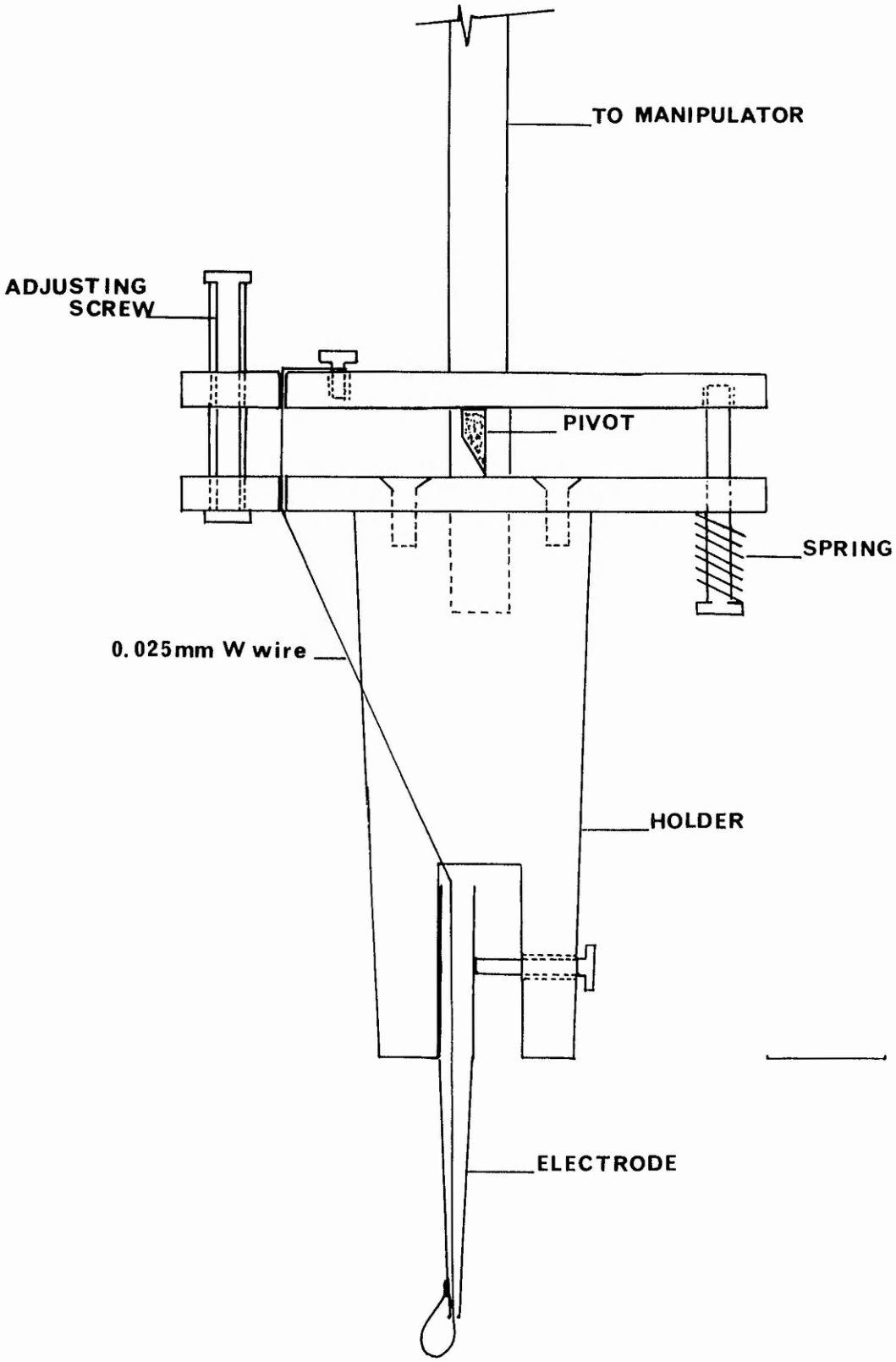
The following chemicals were used (all from BDH unless stated) :- acetylcholine chloride, betaine hydrochloride; choline chloride, eserine (Sigma), 5-hydroxyindole, 5-hydroxyindole acetic acid, 5- hydroxytryptamine creatinine sulphate (Sigma) phosphorylcholine, tryptamine, tryptophan. Acetic acid, n-butanol, ethyleneglycol and formic acid were Analar grade solvents and all inorganic compounds were Analar grade reagents from BDH.

Figure 8.

Design of apparatus constructed for single neurone experiments. The holder, and pivot platforms were made in Perspex and a brass rod was used to attach the whole apparatus to a micromanipulator. See text for details.

Scale bar = 1cm.

(after T.O. Neild, 1975)


TO MANIPULATOR
ADJUSTING SCREW
PIVOT
SPRING
0.025mm W wire
HOLDER
ELECTRODE

Chapter 3.

Intracellular injection of Radiochemicals : Results

3.1. Loss of radioactivity

Radioactivity appeared in the perfusate during and after injection. Experiments were made to determine the extent of loss of radioactivity from injected neurones. Figure 9 shows the results from two experiments when GSC's of Helix pomatia were injected with ^{3}H-5HTP and the appearance of radioactivity in the perfusate was measured. The upper trace of each graph is the corresponding intracellular record at the time of the injection.

In 9.A the neurone is transiently depolarised during the injection of ^{3}H-5HTP (i) but quickly returns to the pre-injection resting potential. (a) and (d) mark the time when the electrode entered and left the bath respectively and (b) and (c) mark the time when the electrode was inserted and removed from the neurone. In 9A the radioactivity first appears when the electrode is placed into the bath (a) falls off while the electrode is in the cell (b-c), rises steeply when the electrode is removed from the neurone (c) and gradually declines to pre-injection levels about 15 minutes after the electrode was removed from the neurone and the bath (d). The results shown for the neurone in 9B show similar pattern, most of the radioactivity appearing just after removal of the electrode (c), but this neurone was more difficult to inject. Four separate injection pulses were required as the electrode tended to come out of the neurone when the pressure was applied. Thus only short duration (<20s) pulses could be used to fill the neurone. The third and fourth injection pulses had much less pronounced effects causing only transient depolarisations. Radioactivity increased in the perfusate throughout the time the electrode was in the neurone reflecting the instability

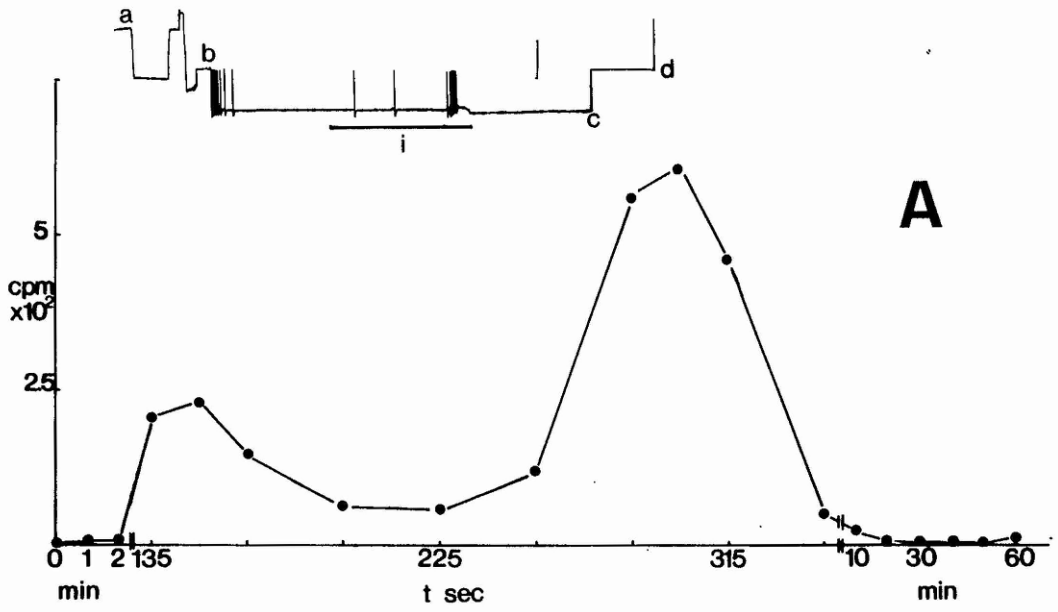
Figure 9.

Loss of radioactivity from Helix pomatia neurones with time following injection of ^{3}H-5-HTP. Upper trace of each graph is the intracellular record of the injected neurone and same time scale applies for the intracellular record and the measurement of radioactivity.

9A : a and d mark the time when the electrode entered (a) and left (d) the bathing solution. b and c are the points when the electrode was inserted and removed respectively from the neurone. i marks the duration of the injecting pulse. This neurone produces a burst of firing when impaled (b) and a slow depolarisation coupled with firing when injected (i). Radioactivity increases when the electrode is placed in the bath and also when it is removed from the neurone.

9B : Same key as 9A. Four separate injection pulses were required as the penetration was much less stable than 9A. Radioactivity increased throughout the injection period although more noticably on removal of the electrode.

Voltage scales for intracellular records :
9A scale bar = 50mV
9B scale bar = 10mV


a
b
d
c
i
A
5
cpm
x10²
2.5
0 1 2 135
225
315
10 30 60
min
t sec
min

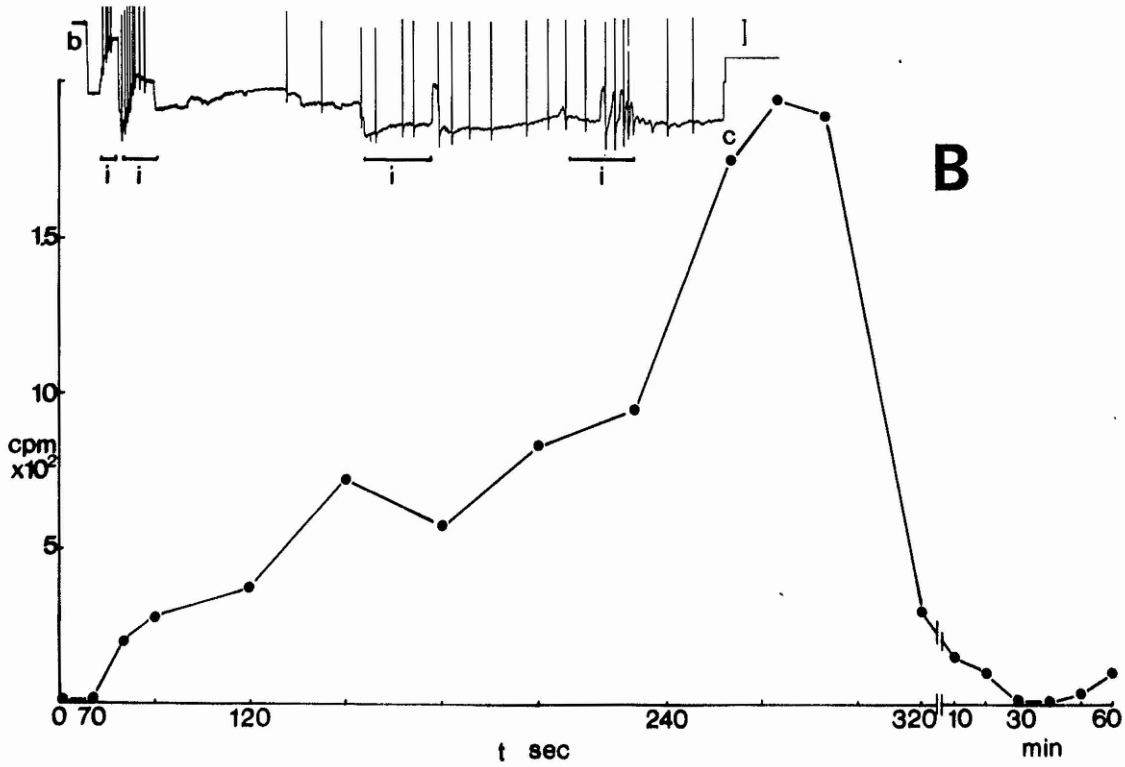

b
i i
i
i
c
B
1.5
10
cpm
x10²
5
0 70
120
240
320 10 30 60
t sec
min

TABLE 3.1. Loss of radioactivity following isotope injection into the GSC s of Helix pomatia.

t (h)	% ^{3}H lost in perfusate after injection of		
	^{3}H-tryptophan	^{3}H-5-HTP	^{3}H-choline
1	40.1 ± 4.2 (4)	35.2 ± 2.2 (3)	17.3 ± 6.6 (8)
6	59.3 ± 8.3 (4)	41.8 ± 2.8 (6)	27.3 ± 5.7 (5h,(4))

Total radioactivity remaining in tissue and total radioactivity in perfusate after 1h and 6h were determined by liquid scintillation counting. The % of ^{3}H in the perfusate was calculated using the following formula : $\frac{^3\text{H in perfusate}}{^3\text{H remaining} + ^3\text{H in perfusate}} \times 100$

TABLE 3.2. Distribution of radioactivity after ^{3}H-tryptophan injection into the GSC s of Helix pomatia.

(h)	total intraneuronal ^{3}H (pmol)	^{3}H-5-HTP (pmol)	^{3}H-5-HT (pmol)	% intraneuronal ^{3}H as ^{3}H-5-HT
1	1.05 - 6.31	Not measured	0.01 - 0.29	3 ± 0.8 (4)
4	1.88 - 9.38	0.06 (1)	0.08 - 0.45	4.7 ± 0.4 (6)
6	1.71 - 3.11	Not measured	0.08 - 0.21	6.6 ± 0.5 (4)
10	0.09 - 14.61	0.21, 0.36	0.004 - 1.28	9.7 ± 1.8 (6)

All percentages are mean ± S.E. (n)

of the penetration.

Table 3.1. summarises the data for the total radioactivity lost in the perfusate one hour and six hours after injection of all three radiochemicals. It can be seen that 40% of the ^{3}H-tryptophan was lost in the first hour and almost 60% in six hours. Analysis of the material in the perfusate indicated that 82% was still in the form of ^{3}H-tryptophan after one hour and 63% after six hours.

There was only a small difference in the ^{3}H loss between one and six hours following ^{3}H-5-HTP injection but whereas 87% of the ^{3}H in the perfusate was identified as ^{3}H-5-HTP after one hour only 36% was in this form after 6 hours. Injected ^{3}H-choline leaked out much less than the other radiochemicals, only 27% in the perfusate after five hours.

3.2. ^{3}H-Tryptophan injection

3.2.(1) Metabolic studies

^{3}H-tryptophan was injected into the Helix pomatia GSC's in amounts ranging from 1.05 to 20.9 pmol. The distribution and form of the radioactivity was determined at 1,4,6 and 10 hours post injection.

Table 3.2. summarises the data from 20 injections and it can be seen that at all times and with all amounts of ^{3}H-tryptophan injected the GSC s synthesised ^{3}H-5-HT. In the 12 experiments where it was measured, ^{3}H-5-HTP was detected in only three and it is interesting to note that the intracellular concentration of ^{3}H-tryptophan in the three neurones was greater than 2.5 mM in each case (see Figure 10).

The percentage of radioactivity associated with 5-HT increases from $3 \pm 0.8\%$ (n = 4) at one hour to $9.7 \pm 1.8\%$ (n = 6) after 10 hours. The maximum amount of ^{3}H-5-HT detected was 1.28 pmol after an initial

Figure 10.

Linear relationship between amount of ^{3}H-tryptophan injected into Helix pomatia GSC s and amount of ^{3}H-5-HT formed. ^{3}H-tryptophan concentration was estimated from a cell volume of 3.2 nl. Regression lines were computed in a HP-25 calculator and $r > 0.9$ in all cases.

The point marked with the star in the 10h experiment is off scale and the coordinates are (4.6 mM , 1.28 pmol ^{3}H-5-HT).

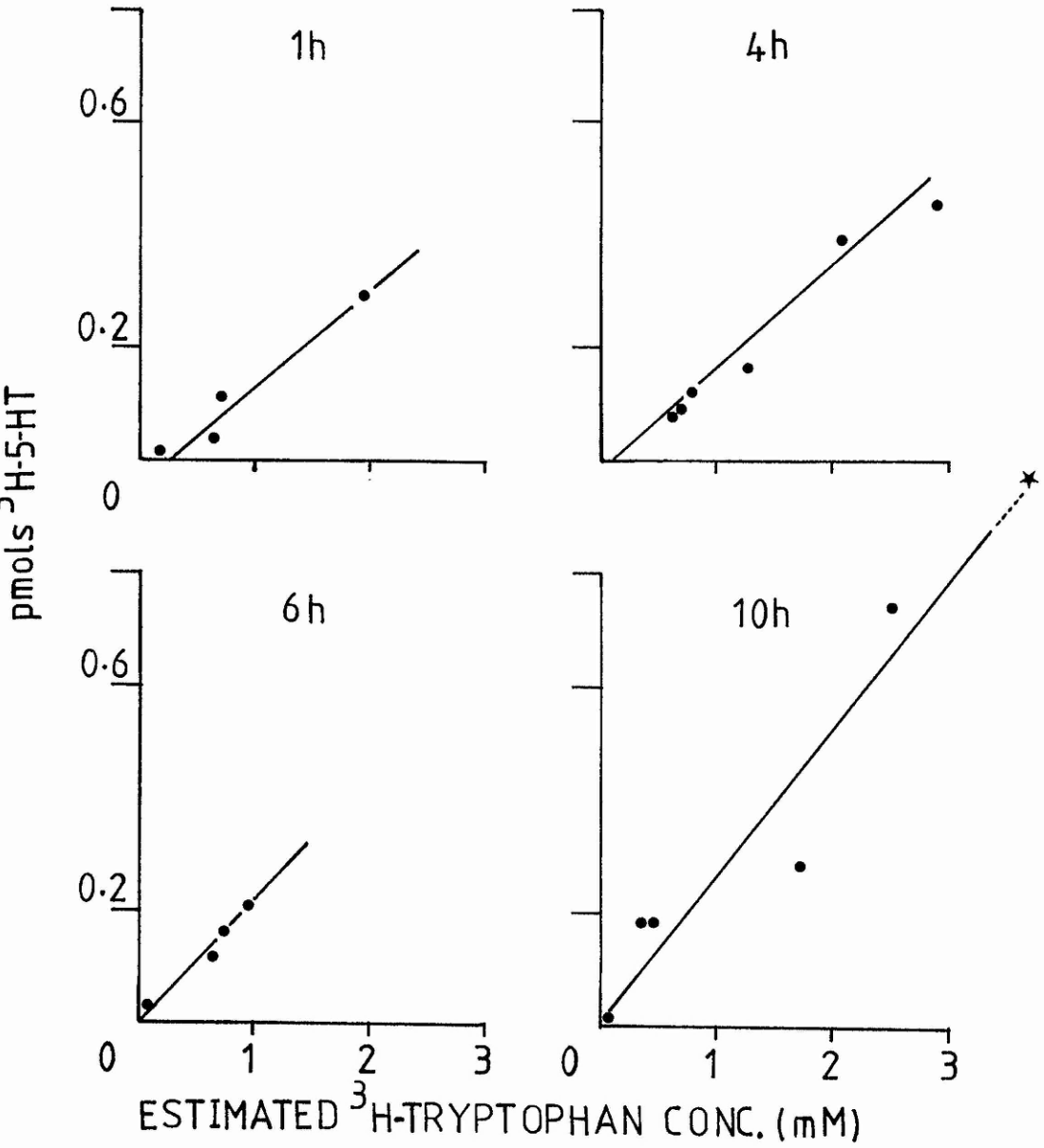

1h
4h
6h
10h
0·6
0·2
0
1
2
3
pmols ^{3}H-5-HT
ESTIMATED ^{3}H-TRYPTOPHAN CONC. (mM)

TABLE 3.3. ^{3}H-5-HT synthesis in identified neurones 4h after ^{3}H-tryptophan injection.

neurone	total intraneuronal ^{3}H (pmol)	^{3}H-5-HT pmol	% intraneuronal ^{3}H as ^{3}H-5-HT
GSC	1.88 - 4.38	0.08 - 0.45	4.7 ± 0.2 (6)
MBC	1.31 - 4.61	0	0 (3)
GVN	1.99, 5.31	0	0 (2)
GSN	0.06, 1.11	0.004, 0.6	6.5, 5.7

All percentages are mean ± S.E. (n)

injection of 36.5 pmol of ^{3}H-tryptophan (only 14.6 pmol of radioactivity remained in the tissue after 10 hours). Intracellular concentrations(mmols . l^{-1}) were calculated on the assumed cell volume of 3.2 nl and when the amounts of ^{3}H-5-HT synthesised were plotted against the estimated intracellular concentrations a good linear relationship at all times was demonstrated. (Figure 10). The process did not appear to saturate at the concentrations studied and even at the highest estimated intracellular concentration (4.6 mM) the process still appeared linear.

The two presumed cholinergic neurones of Helix pomatia, MBC and GVN, do not synthesise either ^{3}H-5-HT or ^{3}H-5-HTP from injected tryptophan (Table 3.3) but the serotonergic neurone of Planorbis corneus produced 0.004 and 0.06 pmol of ^{3}H-5-HT 4 hours after injection of 0.06 and 1.11 pmol ^{3}H-tryptophan, representing 6.5 and 5.7% of the total intraneuronal redioactivity. These latter values are slightly higher than the corresponding values for the Helix pomatia GSC (4.7%) and probably reflect a greater intracellular concentration of ^{3}H-tryptophan in the GSN due to its smaller volume (1nl).

Complete analysis of the intracellular radioactivity in the GSC after four and ten hours and in the MBC, GVN and GSN after four hours is shown in Table 3.4. In the Helix pomatia GSC s there was an increase in the amount of radioactivity which was found in the precipitate from 10.3 $\pm$ 2.6% (n = 4) at one hour (not shown in table) to 17.6 $\pm$ 5.9% (n = 6) after ten hours. There was a similar increase in the unidentifiable portion of the acid soluble fraction which was equivalent to 23.4 $\pm$ 6.1% after ten hours. This acid soluble fraction was not ^{3}H-tryptophan, ^{3}H-5-HTP or ^{3}H-5-HT may have been, in part, ^{3}H-tryptamine. Approximately 3% of the total radioactivity was eluted from chromatograms with tryptamine

TABLE 3.4. % distribution of intraneuronal radioactivity in identified neurones after ^{3}H-tryptophan injection.

Neurone	h	^{3}H-try	^{3}H-5-HTP	^{3}H-5-HT	^{3}H-tryptamine	Other	precipitate
GSC (6)	4	66.1 ± 3	0.1 (1)	4.7 ± 0.4	> 0.3	13.2 ± 2.4	15.6 ± 2.2
GSC (6)	10	46 ± 8.4	2.6, 2.4	9.7 ± 1.8	3.3 ± 0.6	23.4 ± 6.1	17.6 ± 5.9
MBC (3) + GVN (2)	4	71.3 ± 5.6	0	0	0	18.1 ± 5.1	10.6 ± 1.4
GSN (2)	4	57.1, 66.4	2.3, 1.9	6.5, 5.7	0	19.5, 15.9	14.6, 10.1

Where applicable all percentages are mean ± S.E.

Numbers in brackets equal numbers of observations.

(equivalent to 12% of the unidentifiable material). The remainder of this fraction could not be identified. A small amount of ^{3}H-tryptamine was detected in the four hour experiments but this was equivalent to less than 0.3% of the total intraneuronal radioactivity.

After 4 hours more than 70% of the radioactivity in the cholinergic neurones could still be recovered as ^{3}H-tryptophan, as was 66% in the GSC's and approximately 60% in the GSN. The percentage of ^{3}H-tryptophan in the GSCs after ten hours was further reduced to 46%. All neurone types showed a similar incorporation of ^{3}H-tryptophan into the precipitable phase and into the unidentifiable acid extractable phase after four hours suggesting that these processes may be more concerned with metabolic events in the neurones other than transmitter synthesis.

Preincubation, at room temperature for two hours, in physiological solution containing 20 mM parachlorophenylalanine (PCPA) reduced the percentage of radioactivity eluted with 5-HT after six hours (see Table 3.5). One of three PCPA treated GSC s showed no synthesis of ^{3}H-5-HT and in the other two the amount of ^{3}H-5-HT was equivalent to 1.5% of the total intraneuronal radioactivity thus the overall reduction was from 4.4 $\pm$ 0.7% to 1.0 $\pm$ 0.4%. It is interesting to note, however, that the values for the three control brains showed a reduction in the amount of ^{3}H-5-HT synthesised when compared with neurones injected immediately after dissection (4.4 $\pm$ 0.7% c.f. 6.6 $\pm$ 0.5%). This may reflect overall metabolic changes taking place while in vitro.

3.2. (2) Transport of ^{3}H-5-HT in the GSC

After six hours 0.03 to 0.08 pmols of ^{3}H-5-HT could be detected in the buccal ganglia and cerebro-buccal connectives representing 34 $\pm$ 5% (n = 4) of the total intraneuronal ^{3}H-5-HT. No ^{3}H-5-HTP nor

TABLE 3.5. Effects of preincubation with PCPA (20 mM) on ^{3}H-5-HT synthesis in the GSC s of Helix pomatia.

	total interneuronal ^{3}H (pmol)	^{3}H-5-HT pmol	% intraneuronal ^{3}H as ^{3}H-5-HT
Control	0.94 - 3.31	0.04 - 0.16	4.4 $\pm$ 0.7 (3)
PCPA	1.04 - 4.07	0.00 - 0.06	0.8 $\pm$ 0.7 (3)

^{3}H-tryptophan could be detected in the buccal ganglia but in two experiments ^{3}H-tryptophan accounted for 24% and 21% of the radioactivity in the ipsilateral connective. ^{3}H-5-HT accounted for almost 65% of the radioactivity in the buccal ganglia; the remainder was unidentified.

Ten hours post injection a similar amount of the intracellular ^{3}H-5-HT could also be detected in the buccal ganglia and connectives 36.3 $\pm$ 7.7% (n = 6) and again no ^{3}H-tryptophan nor ^{3}H-5-HTP was detected in the buccal ganglia. However in three experiments ^{3}H-tryptophan accounted for 36.7 $\pm$ 6.1% of the ipsilateral connective radioactivity, ^{3}H-5-HT represented 48.1 $\pm$ 3.2% and the remainder was unidentified. ^{3}H-5-HT was responsible for 72. $\pm$ 4.1% of the total radioactivity in the buccal ganglia, the remainder unidentifiable at ten hours.

3.3. ^{3}H-5-Hydroxytryptophan injection

3.3. (1) Metabolic studies

Helix pomatia nervous tissue was analysed at various intervals up to 24 hours after injections of ^{3}H-5-HTP (1.35 - 47.6 pmol) into the serotonin neurones. 0.44 - 5.11 pmol of ^{3}H-5-HT was synthesised by the GSC s under these conditions and at low concentrations (>4 mM) there was a linear relationship between substrate concentration and amount of 5-HT formed. However above 4 mM the process appeared to saturate (see Figure 11).

The percentage of radioactivity eluted with 5-HT increased from 21.9 $\pm$ 0.7% (n = 3) after one hour to almost 28% after ten hours. (Table 3.6.). The seemingly low figure for the four hour experiments is most likely explained by one of the six observations being inexplicably low; this GSC synthesised only 0.11 pmol ^{3}H-5-HT from 1.01 pmol.

Figure 11.

Relationship between intracellular ^{3}H-5-HTP concentration and amount of ^{3}H-5-HT synthesised by Helix pomatia GSC s at various time intervals after injection of ^{3}H-5-HTP. The process appears to saturate above 4 mM ^{3}H-5-HTP which was equivalent to 4 pmol ^{3}H-5-HT.

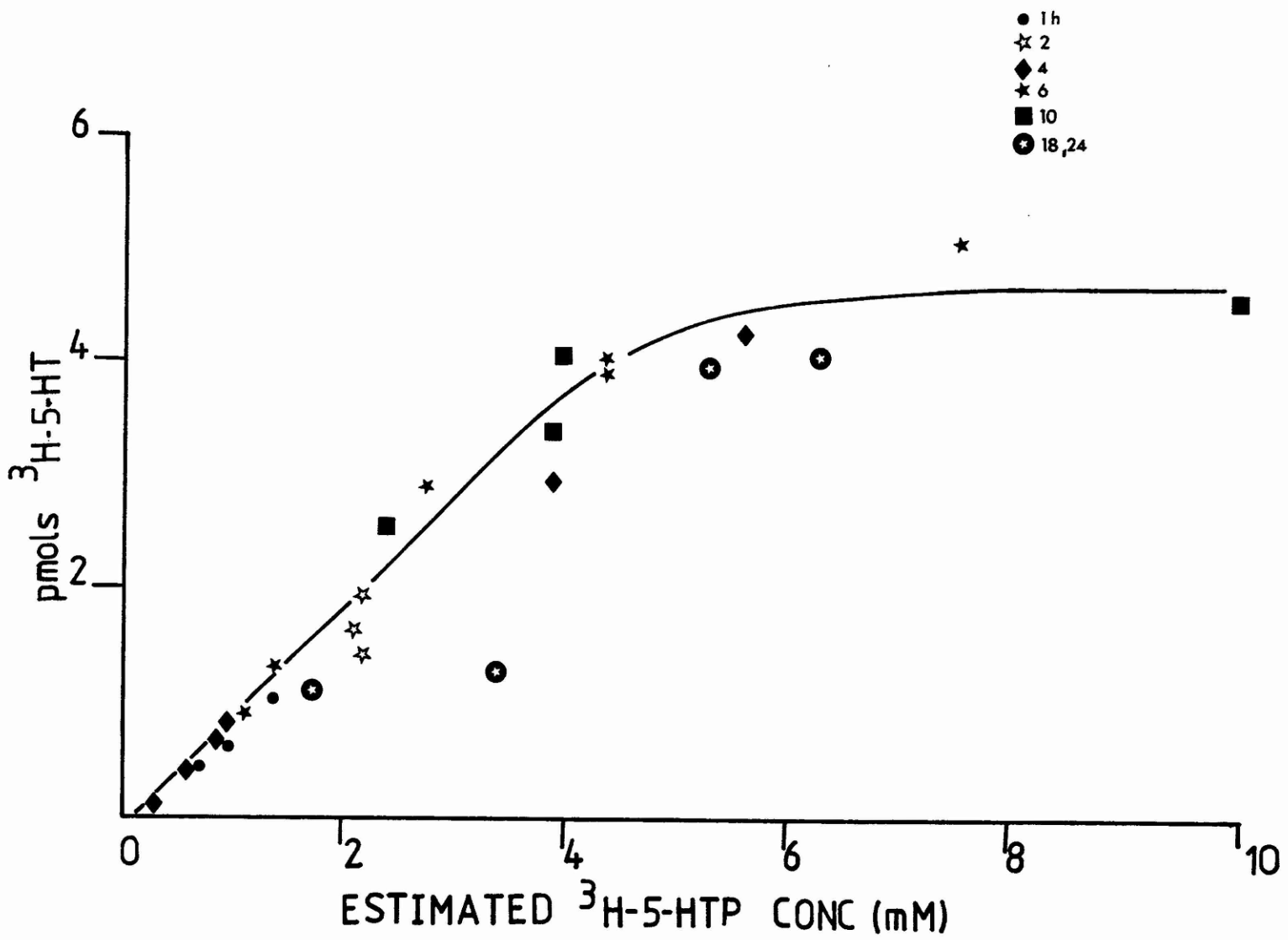

1 h
2
4
6
10
18,24
6
4
2
pmols ^{3}H-5-HT
0
2
4
6
8
10
ESTIMATED ^{3}H-5-HTP CONC (mM)

TABLE 3.6. Distribution of radioactivity after ^{3}H-5-HTP injection into the GSC s of Helix pomatia.

time (h)	total interneuronal ^{3}H (pmol)	^{3}H-5-HT (pmol)	% intraneuronal ^{3}H as ^{3}H-5-HT
1	2.01 - 4.32	0.44 - 1.01	21.9 ± 0.7 (3)
2	6.61 - 6.92	1.56 1 1.91	23.6 ± 2.2 (3)
4	1.01 - 17.63	0.11 - 4.25	22 ± 2.2 (6)
6	3.60 - 24.17	0.90 - 5.11	27.6 ± 1.5 (6)
10	7.33 - 32.40	2.65 - 4.50	27.9 ± 4.3 (4)
18	14.66, 20.01	3.99, 4.00	27.2, 20
24	5.39, 10.66	1.14, 1.29	21.2, 12.1

TABLE 3.7. Distribution of radioactivity 6 hours after injection of ^{3}H-5-HTP into (i) GSC and (ii) neuropil of Helix pomatia.

	% ^{3}H retained in tissue	% intraneuronal ^{3}H as ^{3}H-5-HTP	^{3}H-5-HT
GSC (6)	58.2 ± 2.8	41.1 ± 4.7	27.6 ± 1.5
Neuropil (3)	14.8 ± 4.0	52.5 ± 4.2	4.1 ± 1.9

All precentages are mean ± S.E. (n)

^{3}H-5-HTP, equivalent to a conversion of 10.9% whereas the mean conversion of the remaining five observations was 24.3 $\pm$ 0.9%. There is an apparent decline in the amount of ^{3}H-5-HT after ten hours but there is insufficient data for statistical significance. Although 3 and 4 neurones were injected satisfactorily and left 18 and 24 hours respectively only 2 cells from each group had satisfactory resting potentials at the end of the experiment.

In order to ensure that the ^{3}H-5-HT was derived from intraneuronal enzymatic synthesis within the GSC and not from ^{3}H-5-HTP which had leaked from the neurone and had been reuptaken elsewhere, three experiments were made where the substrate was injected directly into the neuropile of one cerebral ganglion. Analysis of the tissue 4 hours after the injection showed that less than 15% of the injected radioactivity had been retained in the tissue (Table 3.7). Most of the ^{3}H was lost during injection and in the first ten to fifteen minutes after removal of the electrode. Of the radioactivity retained by the nervous tissue only 4.1 $\pm$ 1.9% was in the form of ^{3}H-5-HT after 6 hours and 52.5 $\pm$ 4.2 was still ^{3}H-5-HTP. The corresponding figures for intraneuronal injection were 27.6 $\pm$ 1.5 and 31.1 $\pm$ 4.7 for ^{3}H-5-HT and ^{3}H-5-HTP respectively. Thus only a small proportion of the endogenous ^{3}H-5-HT is likely to have arisen from leaked ^{3}H-5-HTP.

The proportion of intraneuronal radioactivity associated with ^{3}H-5-HTP showed a decrease with time concomitant with the increase in ^{3}H-5-HT. (Table 3.8.) but never fell lower than 35%. The proportion of radioactivity associated with the non acid-extractable precipitate increased with time from 3.6 $\pm$ 1.1 to 10.2 $\pm$ 1.7% 10 hours and to around 20% after this time (Table 3.8). The unidentifiable fraction remained near the origin during electrophoresis and a portion of this fraction

TABLE 3.8. % distribution of intraneuronal radioactivity after ^{3}H-5-HTP injection into the GSC s of Helix pomatia.

t (h)	^{3}H-5-HTP	^{3}H-5-HT	Other	precipitate	
1	63.1 ± 1.3	21.9 ± 0.7	11.4 ± 1.1	3.6 ± 1.1	(3)
2	55.2 ± 4.6	23.6 ± 2.2	14.8 ± 4.2	6.4 ± 1.8	(3)
4	44.4 ± 4.8	22 ± 2.2	26.9 ± 3.8	6.8 ± 1.2	(6)
6	42.4 ± 2.0	27.6 ± 1.5	23.2 ± 2.8	6.7 ± 1.2	(6)
10	38.6 ± 1.3	27.9 ± 4.3	23.4 ± 6.0	10.2 ± 1.7	(4)
18	40.1 , 44.3	27.2, 20	12.3, 24.4	20.4, 11.3	
24	36.7, 35.4	21.2, 12.1	24.5, 29.1	17.6, 33.4	

All percentages are mean ± S.E. (n).

TABLE 3.9. ^{3}H-5-HT synthesis in identifiable neurones 6 hours after ^{3}H-5-HTP injection.

	total intraneural ^{3}H pmol	^{3}H-5-HT (pmol)	% intraneuronal ^{3}H as ^{3}H-5-HT
GSC	3.6 - 24.17	0.90 - 5.11	27.6 ± 1.5 (6)
MBC	5.94 - 16.31	0	0 (3)
GVN	4.26, 7.53	0	0 (2)
GSN	0.79 - 2.31	0.24 - 0.45	29.4 ± 4.5 (3)
GDC	0.81 - 4.66	0.09 - 0.86	15.8 ± 1.9 (3)

may have been in the form of 5-HIAA.

The cholinergic neurones lack the ability to convert ^{3}H-5-HTP into ^{3}H-5-HT (Table 3.9) whereas the serotonin neurone (GSN) and the dopamine neurone (GDC) of Planorbis both are able to synthesise ^{3}H-5-HT from its precursor (Table 3.9). The percentage converted in the GSN is similar to the figure for the GSC but the GDC value is almost 50% less than values of the serotonin cells.

3.3. (2) Transport of radioactivity

^{3}H appeared in the ipsilateral cerebrobuccal connective and internal and external lip nerves within an hour of ^{3}H-5-HTP injection (Table 3.10). The radioactivity first appeared in the ipsilateral buccal ganglion at four hours at which time it also could be detected in the contralateral lip nerves and connective. There was a progressive movement of radioactivity from the cerebral ganglia to the nerves, connectives and buccal ganglia and after 10 hours more than 30% of the radioactivity had moved from the injection site. Radioactivity in the buccal ganglia accumulated much more rapidly than in the connectives and lip nerves (from 0-18% total in 10 hours compared with from 7% to 16% in the same period for the connectives and lip nerves).

The fraction of the intraneuronal radioactivity represented by ^{3}H-5-HT showed a similar distribution pattern to the total radioactivity. For example, after one hour 90% of the total ^{3}H-5-HT was located in the cerebral ganglion (Table 3.11) but after 10 hours 18% could be found in the nerves and connectives and almost 26% in the buccal ganglia. When the latter figure is compared with the figure for the percentage of total radioactivity in the buccal ganglia (18%, Table 3.10) it can

TABLE 3.10. Distribution of intraneuronal radioactivity following ^{3}H-5-HTP injection into the GSC s of Helix pomatia.

time	IPSILATERAL				CONTRALATERAL		
	Cerebral Ganglia	connective	Lip nerves	buccal ganglion	connective	Lip nerves	buccal ganglion
1 (3)	93 ± 3.2	4.2 ± 2.1	2.7 ± 1.2	0	0	0	0
4 (3)	83.4 ± 4	6.5 ± 1.4	2.6 ± 0.8	3.6 ± 1.0	1.8 ± 0.8	2.3 ± 0.8	0
6 (6)	77 ± 3.6	7.8 ± 1.5	3.4 ± 1.4	5.9 ± 0.7	1.9 ± 0.3	1.2 ± 0.3	2.3 ± 0.5
10 (4)	66.3 ± 4.2	7.2 ± 3.1	5 ± 1.4	12.1 ± 0.5	2 ± 0.5	2.2. ± 0.9	5.9 ± 0.7

All percentages are mean ± S.E. (n)

TABLE 3.11. % distribution of intraneuronal ^{3}H-5-HT following ^{3}H-5-HTP injection into the Helix pomatia GSC s.

t (h)	Cerebral Ganglia	Nerves and Connectives	Buccal Ganglia
1	90.4	9.4	0
4	75.5	13.9	9.1
6	63.8	17.8	17.8
10	55.4	17.9	25.8

TABLE 3. 12. % total intraneuronal ^{3}H as ^{3}H-5-HT in :

t (h)	Cerebral Ganglia	Connective	Nerve	Buccal Ganglion
1	21.3	21.4	44.4	0
4	22.4	40	34.5	63.9
6	19.9	37.2	58.8	83.1
10	17.4	41.3	47.6	59.5

be seen that there is a preferential relocation of the intraneuronal ^{3}H-5-HT. This observation is also supported by the data displayed in Table 3.12 where it can be seen that in the connective, lip nerves and buccal ganglia ^{3}H-5-HT represents a much higher percentage of the total intraneuronal radioactivity than in the cerebral ganglia. Thus the "enrichment" of these tissues with ^{3}H-5-HT indicates a specific transport mechanism for the amine.

3.3. (3) Transport of radioactivity in the ipsilateral cerebrobuccal connective

Analysis of 1 mm segments of ipsilateral cerebrobuccal connectives at varying time intervals after ^{3}H-5-HTP injection showed that the radioactivity was transported down the connective in a series of peaks and troughs (Figure 12). An estimate of the fastest movement is 2 to 3 mm.hr^{-1} based on the observation that radioactivity first appeared in the buccal ganglion at 4 hours and in the connective in less than 1 hour and the mean length of the connective is approximately 8 to 12 mm. Chromatographic and electrophoretic analysis of the initial three segments and the final three segments from 4 "pooled" experiments 6 hours after injection showed that 77% of the radioactivity in the initial segments was ^{3}H-5-HTP and 16.5% was ^{3}H-5-HT whereas ^{3}H-5-HT in the final segments accounted for 81.4% of the total radioactivity.

3.3. (4) Release of radioactivity

6 hours after ^{3}H-5-HTP injection into one GSC of three animals the neurones were stimulated antidromically by a suction electrode attached to the internal lip nerve. The fluid perfusing the buccal ganglia was collected in half hour periods and analysed.

Although the physiological solution had been supplemented with imipramine (10^{-5}M) only small amounts of radioactivity could be detected

Figure 12.

Distribution of radioactivity in 1 mm segments of the ipsilateral cerebrobuccal connective at various times after intrasomatic injection of ^{3}H-5-HTP into the giant serotonin neurone of Helix pomatia.

Ordinate : Radioactivity per segment expressed as a fraction of the total radioactivity in the ipsilateral cerebral ganglion, the ipsilateral connective and the buccal ganglia (see inset diagram). The data is "normalised" in this way to minimise any differences due to different amounts of injected ^{3}H-5-HTP.

The expanded scales are required since more than 60% of the radioactivity remains within the cerebral ganglia. Same values apply to all the expanded scales (see 1h) and actual values are given on each graph.

Abscissa : 1 mm segments of ipsilateral cerebrobuccal connective.

The arrows mark peaks or shoulders which may correspond to moving fronts.

The inset (6h experiment) is a diagramatic representation of the relative positions of the cerebral ganglia (CG), cerebrobuccal connective (CBC) and buccal ganglia (BG).

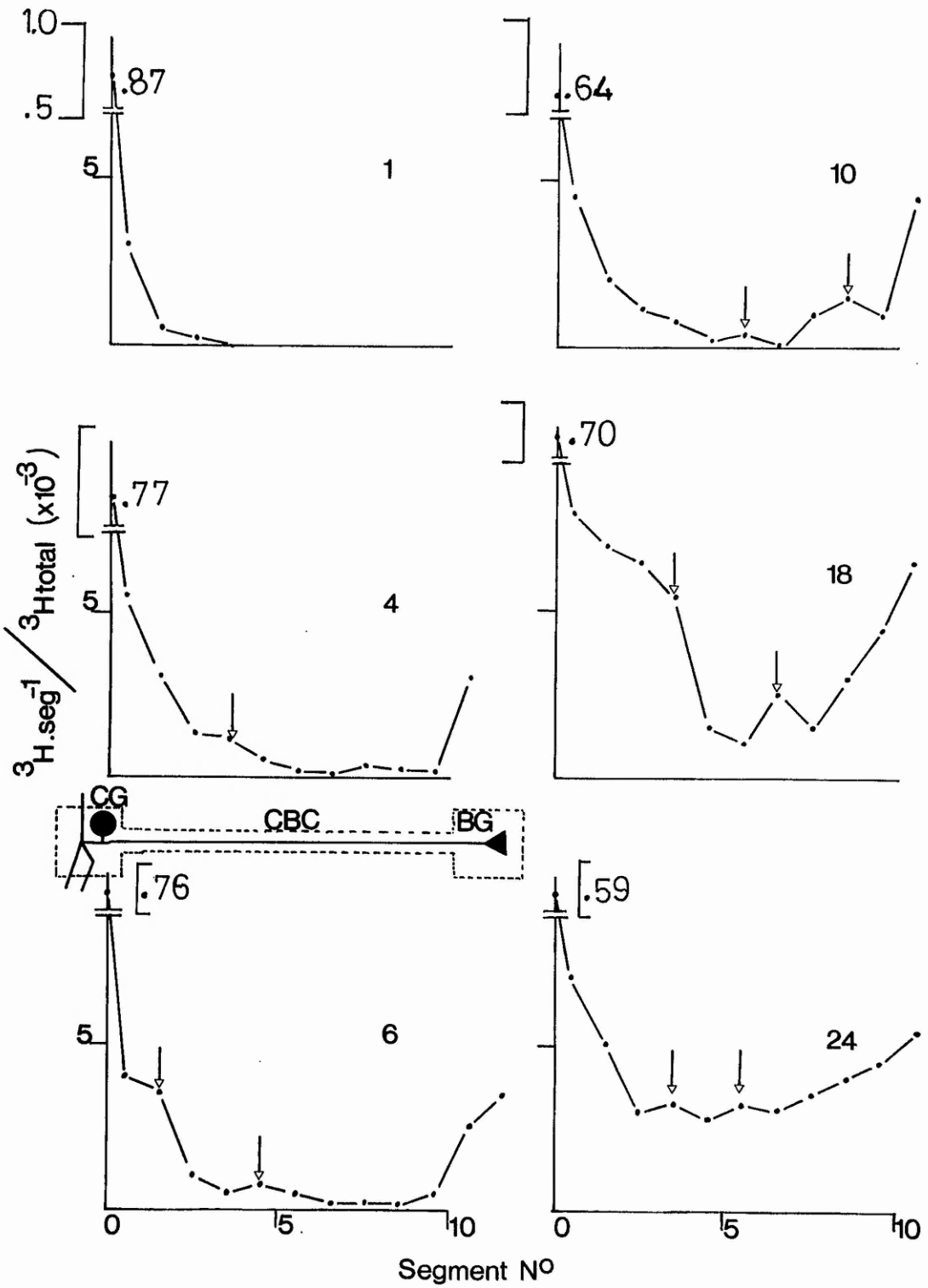

1.0
.5
.87
5
1
.64
10
$^3H.seg^{-1}$ / $^3H_{total}$ ($x10^{-3}$)
.77
5
4
.70
18
CG
CBC
BG
.76
5
6
.59
24
0
5
10
0
5
10
Segment N°

Figure 13.

Release of radioactivity from terminals of the Helix pomatia serotonin neurones after intrasomatic injection of ^{3}H-5-HTP at time zero.

Each bar represents mean $\pm$ S.E.M. for three neurones. Solid block represents a 15 minute period of electrical stimulation of the GSC. The amount of radioactivity released was rationalised to the amount injected.

There is a progressive increase in the radioactivity in the perfusate after three hours and stimulation causes an increase in the amount released.

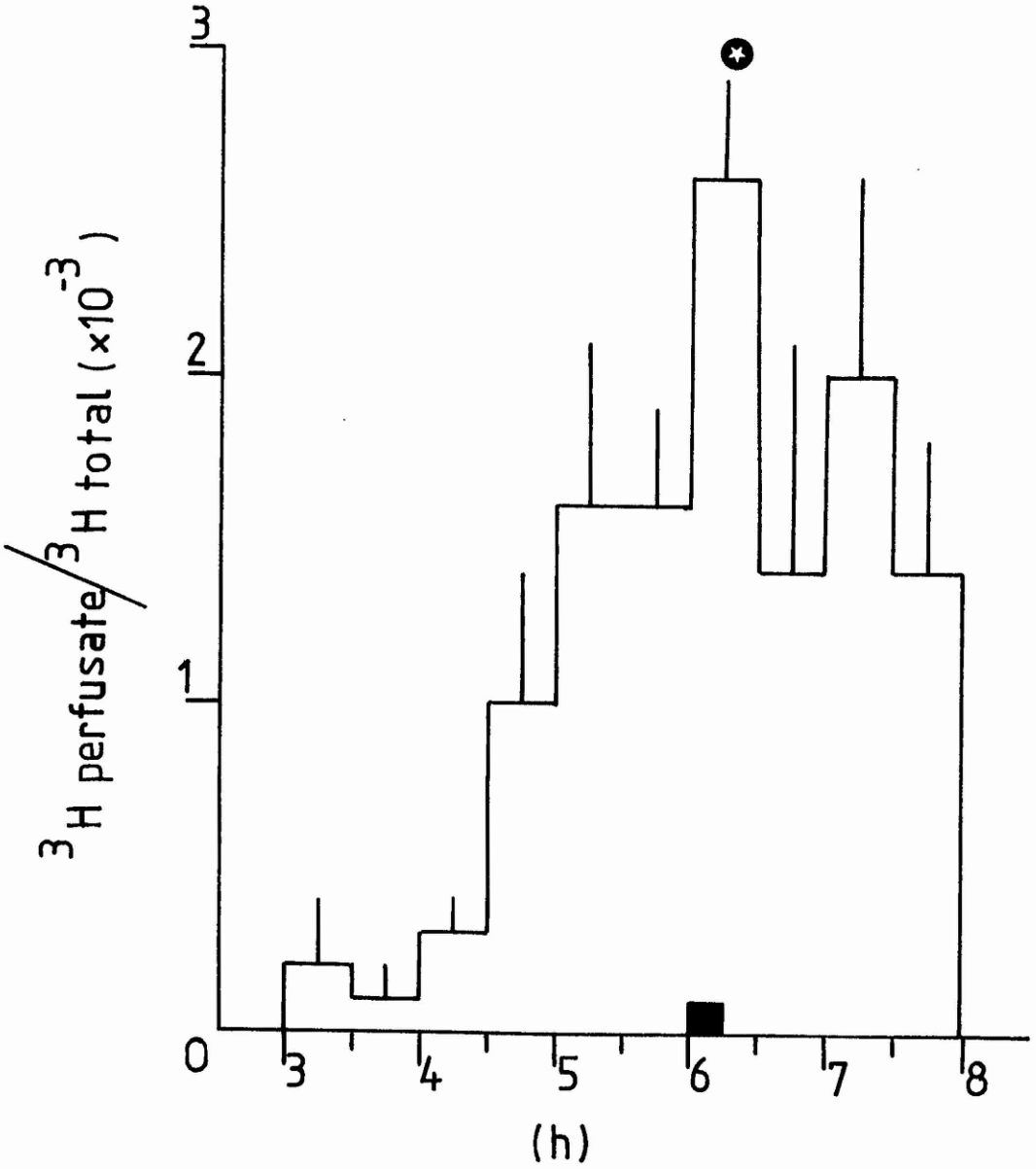

^{3}H perfusate/^{3}H total (×10^{-3})
3
2
1
0
3
4
5
6
7
8
(h)

TABLE 3.13. Synthesis of ^{3}H-ACh after ^{3}H-choline injection in the GSC s of Helix pomatia.

t (h)	total intraneuronal ^{3}H (pmol)	^{3}H-ACh (pmol)	% intraneuronal ^{3}H as ^{3}H ACh
1	1.12 - 29.34	0.07 - 6.11	12 ± 0.5 (8)
5	5.41 - 16.45	0.76 - 2.63	13.8 ± 1.1 (4)
10	2.97 - 15.30	0.19 - 1.57	10.3 ± 21 (4)

TABLE 3.14. Distribution of intraneuronal ^{3}H after ^{3}H-choline injection.

t (h)	^{3}H-choline	^{3}H-ACh	^{3}H (PC + Betaine)	Other	
GSC 1	42.5 ± 4.1	12.0 ± 0.5	36.7 ± 1.7	8.8 ± 0.7	(8)
GSC 5	27.5 ± 2.0	13.8 ± 1.1	49.2 ± 2.7	9.5 ± 1.4	(4)
GSC 10	18.8 ± 0.6	10.3 ± 2.1	57.1 ± 2.4	13.8 ± 0.9	(4)
MBC 1	8.3 ± 0.4	55 ± 2.5	25.8 ± 2.9	10.9 ± 0.6	(5)

All percentages are mean ± S.E. (n)

before, during and after stimulation. The maximum being equivalent to approximately 30 fmols ^{3}H-5-HT released in the 30 min period which included the stimulation. When the amount of ^{3}H in the perfusate is rationalised to the total amount in the tissue and the mean of the three experiments taken, a histogram as shown in Figure 13 is obtained. Although all three neurones showed an increased release over the previous time period the mean differences were not statistically significant (paired t-test). However it is interesting to note that although the increase in two of the neurones was less than 30% of the pervious period the third cell exhibited a 71% increase in released radioactivity. In all three experiments the amount released was insufficient for chemical analysis.

3.4. ^{3}H-Choline injection

3.4. (1) Metabolic studies

1.37 to 29.34 pmol ^{3}H-choline were injected into the Helix pomatia GSC's and the tissues analysed 1,5 and 10 hours later.

The GSC's converted 12% of the ^{3}H-choline to ^{3}H-ACh after 1 hour (Table 3.13), and 13.8 $\pm$ 1.1% and 10.3 $\pm$ 2.1% after 5 and 10 hours. The maximum amount of ^{3}H-ACh detected was 6.11 pmol . There was a linear relationship between the amount of ^{3}H-ACh synthesised and the intracellular concentration of ^{3}H-choline ($r = 0.83$) (see Figure 14) up to approximately 2.5 mM. Thereafter with increasing concentration there was a great variation in the amounts of ^{3}H-ACh synthesised although if the 6.11 pmol result is ignored the process may saturate at 2.5 mM choline which corresponded to approximately 1 pmol ^{3}H ACh synthesised/cell/h.

The radioactivity not associated with ^{3}H-ACh was eluted with choline, phosphorylcholine (PC) and betaine, and a non extractable portion. The proportion of radioactivity associated with PC and betaine increased with time concomitant with a decrease in choline (Table 3.14).

Approximately 45 per cent of the acid non extractable

Figure 14.

Dependence of ^{3}H-ACh synthesis on the amount of injected ^{3}H-choline in identified Helix pomatia neurones one hour after injection.

Upper graph : Data from injected GSC's.
Normal or intact GSC's (circles) and isolated (lig, stars) exhibit a linear relationship between substrate concentration and amount of enzyme activity at low substrate concentrations. Above 2 mM the three "normal" values show a great variance.

Lower graph : Data from injected cholinergic neurones.
Five medial buccal neurones (MBC, squares) and three giant visceral neurones (GVN, circles) show a good linear relationship between substrate concentration and enzyme activity. The process does not saturate at the concentrations of ^{3}H-choline used.

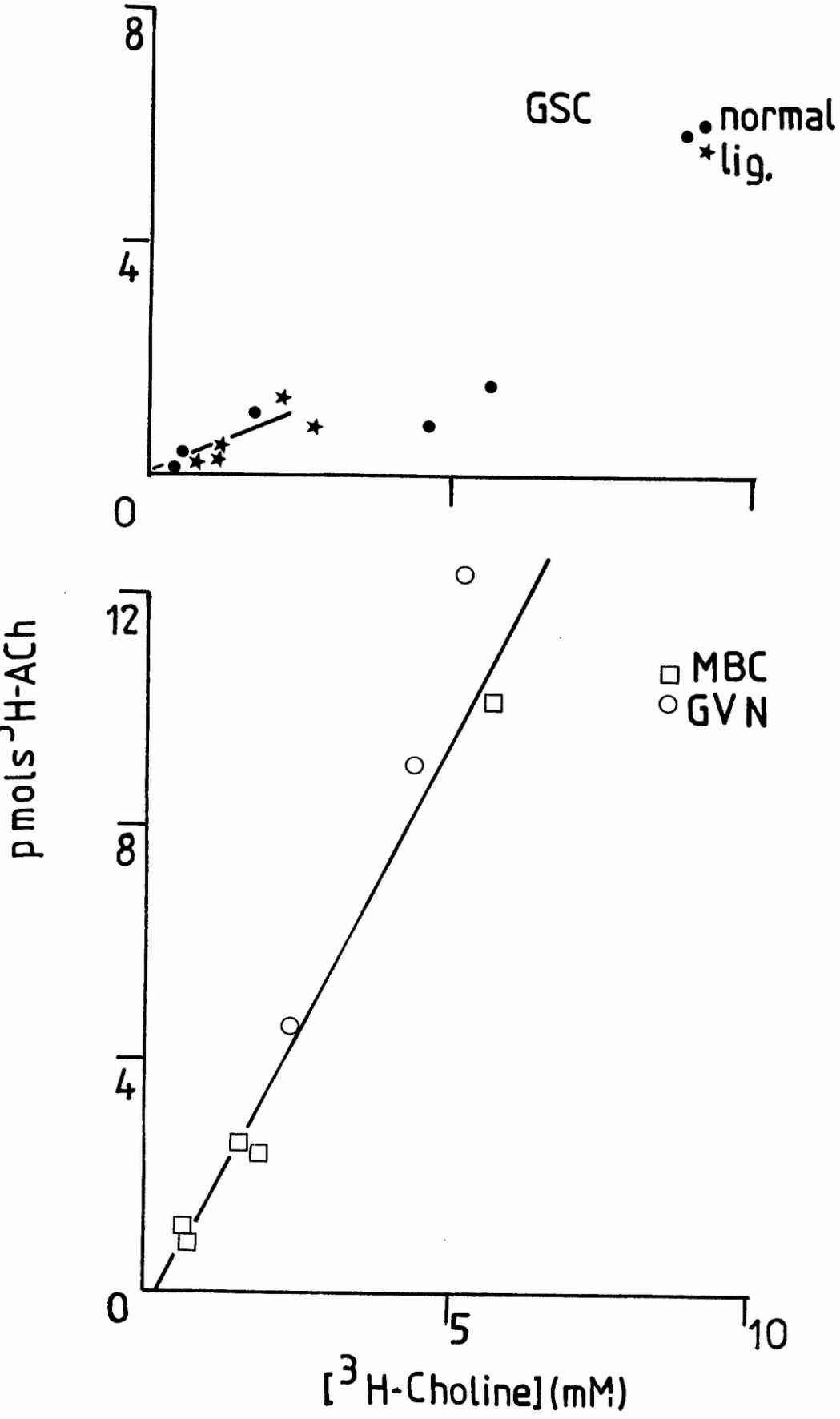

8
4
0
GSC
normal
lig.
12
8
4
0
5
10
pmols ^{3}H-ACh
MBC
GVN
[^{3}H-Choline] (mM)

TABLE 3.15. ^{3}H-ACh synthesis 1 hr after ^{3}H-choline injection into identifiable neurones.

	total intraneuronal ^{3}H (pmol)	^{3}H-ACh pmol	% intraneuronal ^{3}H as ^{3}H-ACh
1. Serotonin neurones			
GSC (H. pomatia)	1.12 - 29.34	0.07 - 6.11	12 ± 2.9 (8)
GSC (H. aspersa)	2.16 - 7.26	0.38 1 0.91	15 ± 2.6 (3)
GSN (P. corneus)	4.35 - 10.40	0	0 (3)
2. Cholinergic neurones			
MBC (H. pomatia)	1.74 - 16.56	1.05 - 10.11	55 ± 2.5 (5)
GVN (H. pomatia)	7.27 - 16.41	4.55 - 12.47	68.5 ± 0.9 (3)
3. Dopamine neurone			
GDC (P. corneus)	5.76, 6.44	0, 0	0
4. Neuropil			
(H. pomatia)	11.46, 20.11	0.58, 1.06	5.1, 5.3

radioactivity could be extracted from the precipitate in methanol/chloroform representing the ^{3}H-choline incorporated into lipid.

^{3}H-choline injection into the homologous GSC's of Helix aspersa also induced synthesis of ^{3}H-ACh, equivalent to a conversion of 15 $\pm$ 2.6% of the total intraneuronal radioactivity. The two identifiable amine containing neurones of Planorbis were unable to covert ^{3}H-choline to ^{3}H-ACh (table 3.15) thus they appear to lack the enzyme ChAT.

The amount of ^{3}H-ACh recovered from Helix CNS after injection of ^{3}H-choline into either of the presumed cholinergic neurones was dependent on the amount injected. (Figure 14). After one hour the MBC converted 55% of the ^{3}H-choline to ^{3}H-ACh and the GVN had converted almost 70%. In both neurones less than 10% of the radioactivity could still be extracted as ^{3}H-choline and less than 2% was in the form of lipid. The remainder was betaine and phosphorylcholine.

The Helix pomatia neuropil was able to synthesise up to 1.06 pmol of ^{3}H-ACh following injection of choline directly into the neuropil. (Table 3.15). This corresponded to a conversion of 5%.

3.4. (2) Transport of ^{3}H after ^{3}H-choline injection into the GSC's

Radioactivity appeared in the cerebro-buccal connective within 1 hour after injection of ^{3}H-choline, 5 hr post injection 7.2 $\pm$ 3.6% (n = 4) of the total radioactivity was detected in the buccal ganglia. Analysis of this material showed that 42% of the buccal ganglion radioactivity was ^{3}H-ACh compared with 11.3% in the cerebral ganglia and 17.5% in the nerves and connectives.

In four experiments the cerebro-buccal connectives were ligated close to their exits from the cerebral ganglia, as were the contralateral lip nerves. All three ipsilateral lip nerves were ligated as

far as possible from their exits from the ganglia. When radioactivity in the three nerves was analysed 3 hours after the injection the middle lip nerve, in all experiments, contained no radioactivity. The internal lip and external lip nerves contained 17.6 $\pm$ 4.9% of the total intraneuronal ^{3}H and 34.5% of this was in the form of ^{3}H-ACh. Thus confirming that the radioactivity is confined to nerves which contain axons of the GSC and does not appear to result from reuptaken choline being converted in other neurones.

3.4.(3) Transport of ^{3}H in the ipsilateral cerebro-buccal connective

2,4,6 and 10 hours after injection of ^{3}H-choline the ipsilateral cerebrobuccal connective was sectioned into 1 mm segments and the radioactivity in each segment was counted. The results from four neurones are shown in Figure 15. Unlike the corresponding experiments after ^{3}H-5-HTP injection there are much fewer peaks and troughs in the profiles of radioactivity in the cerebro-buccal connectives. However the increase in the radioactivity in the buccal ganglia suggests that the ^{3}H is accumulating here. Analysis of proximal and terminal segments (each 3 mm long) pooled from a further three injected neurones showed that the proportion of radioactivity associated with ^{3}H-ACh was increased only slightly from 17.5% to 20.2%. Nevertheless, these figures should be compared with 11.3% as the amount of ^{3}H-ACh in the cerebral ganglia and 42% in the buccal ganglia and this suggests that there may be a preferential transport of ^{3}H-ACh from the neuron soma but that there may also be a mechanism for synthesising ^{3}H-ACh in the terminal region of the serotonin neurones or for accumulating ^{3}H-ACh.

3.4.(4) Release of radioactivity in the buccal ganglia

6 hours after injection, the GSC's were stimulated in an

Figure 15.

Distribution of radioactivity in 1 mm segments of ipsilateral cerebro-buccal connective at various times after intrasomatic injection of ^{3}H-choline into the giant serotonin neurone of Helix pomatia.

(see Figure 12 for details)

Points off scale on the ordinate axis are equal to 0.84 (2h); 0.79 (4h); 0.61 (6h); 0.67 (10h).

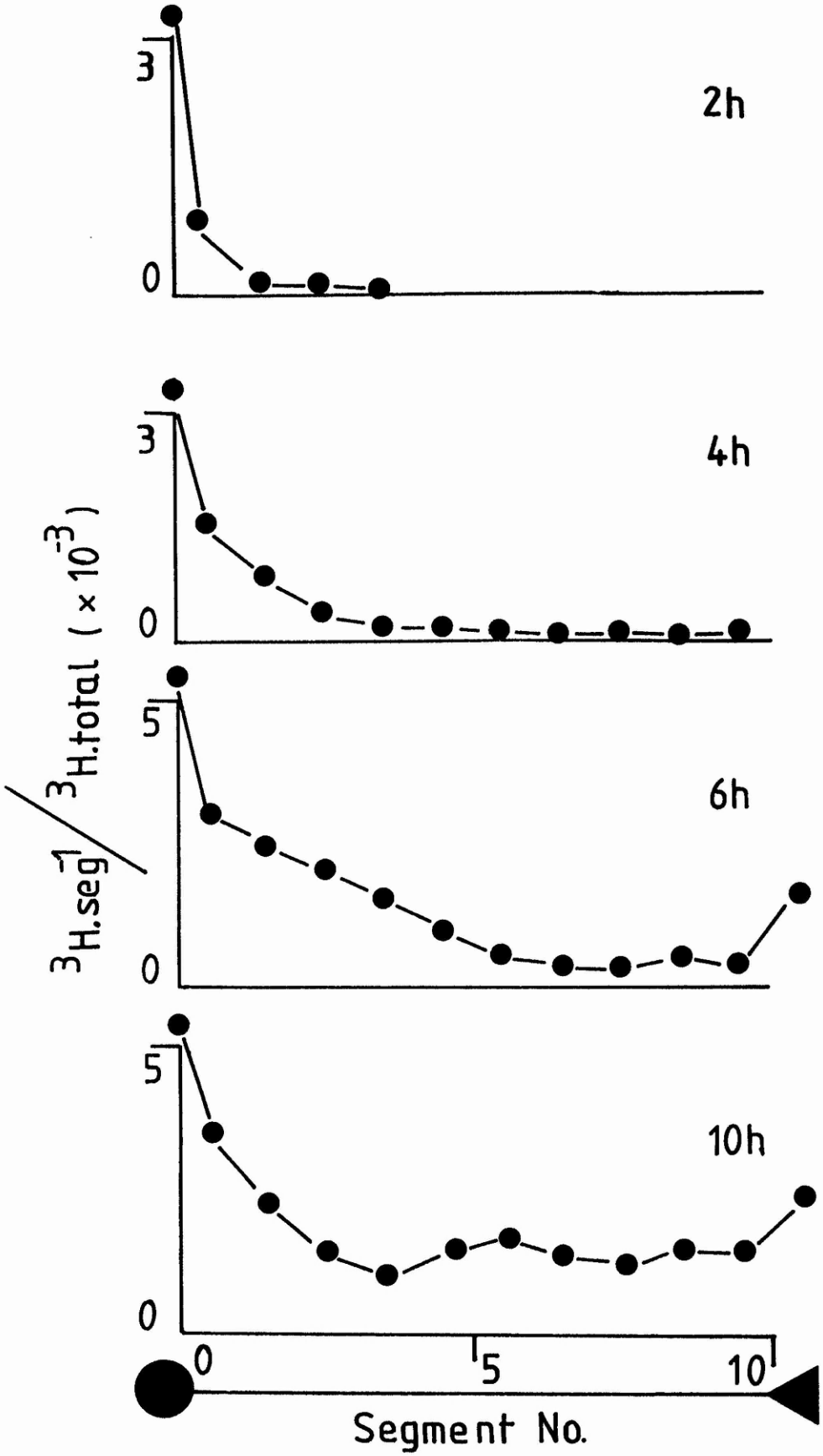

2h
4h
6h
10h
3
0
5
$^3H.seg^{-1}$ / $^3H.total$ ($\times 10^{-3}$)
0
5
10
Segment No.

TABLE 3.16. Release of radioactivity after ^{3}H-injection into the *Helix pomatia* GSC *s*.

Collection* period	total** cpm	^{3}H-ACh cpm	Unknown cpm	^{3}H-choline cpm	^{3}H-choline cpm.h^{-1}
(i)	101	N.D	35	76	38
(ii)	84	N.D	60	24	48
(iii)	124	N.D	46	78	39

N.D = None detected

* see text for details

** total from 6 pooled experiments

identical manner to that described in 3.3.(4). Radioactivity was collected and analysed as also described in 3.3.(4). No radioactivity was detected up to, during and after the stimulation period in two out of four experiments. In the remaining two experiments no radioactivity was detected up to the stimulating period, then 11 cpm and 23 cpm were detected in this period. These results are equivalent to 1.8 and 3.9 fmoles of radioactivity and were uncharacterised biochemically. No further radioactivity was detected in the subsequent periods.

In an attempt to further characterise the released material six neurones were injected, left for 6 h and stimulated for 15 minutes as described before. In each experiment the buccal ganglia perfusate was collected for the following intervals (i) from injection to stimulation (6h), (ii) stimulating period plus 15 minutes (0.5h) (iii) post stimulus period (2h). In each experiment the bathing fluid contained 10^{-3}M eserine. The samples from each experiment were pooled, reduced in vacuo and subjected to electrophoresis.

The data are summarised in table 3.16. There is an increase in the radioactivity released during the stimulation period from 50 to 101 cpm.h^{-1} (assuming radioactivity present in the buccal ganglia only after 4-5 hours) to 168 cpm.h^{-1} followed by a reduction in the post-stimulus period. None of the released material was identified as ^{3}H-ACh but there was a measurable increase in the amount of ^{3}H-choline released during the stimulation period. The appearance of an unidentified substance in the perfusate also increased during stimulation.

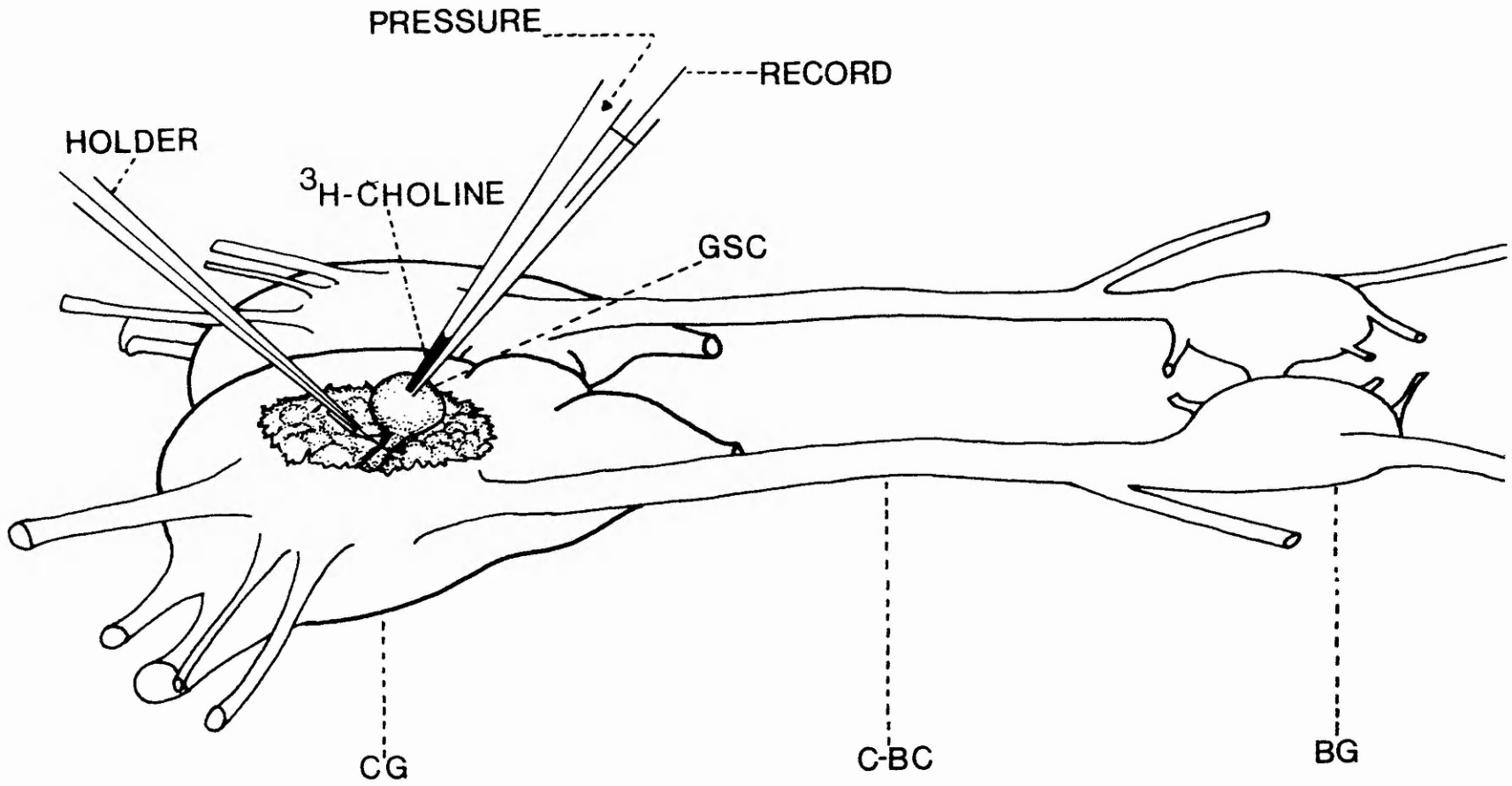
3.4. (5) Single neurone experiments

As a final check to ensure that endogenous ^{3}H-ACh was

Figure 16.

Diagram of experimental arrangement for single neurone experiments. The GSC's were freed from the surrounding tissue, lassoed by the wire loop and held firmly for injection. (Technical details in text, 2.2. (9)).

BG, buccal ganglia; C-BC, cerebrobuccal connective; CG, cerebral ganglia.


PRESSURE
RECORD
HOLDER
^{3}H-CHOLINE
GSC
CG
C-BC
BG

not derived from any sources other than the GSC, fifteen experiments were made using the experimental design shown in Figure 15. Five GSC's were injected and after 1 hour the tissue was analysed as normal. Figure 16 shows two representative electropherograms which clearly show a peak corresponding to ACh (A). In one experiment half of the acid extract containing the radioactivity was heated in a water bath to 40° for 5 mins after the addition of 1 M NaOH. The extract was then reacidified and chromatographed along-side the untreated portion. Figure 17 shows that the shoulder on the choline peak which is probably ^{3}H-ACh is considerably reduced after the alkali and heat treatment suggesting that the product is in fact ^{3}H-ACh. The five GSC's in these experiments converted a similar amount of ^{3}H-to ^{3}H-ACh (11.6 $\pm$ 2.4%) as the normal neurones although the range of ^{3}H-choline injected was much less (2.31 - 8.66 pmols).

In a further 5 experiments the neurones were left for 5 hours post injection after which the buccal ganglia, connectives and nerves were analysed for radioactivity. In one of the five experiments no radioactivity was detected in the nerves or buccal ganglia but in the other four, 3.9 $\pm$ 1% of the total intraneuronal radioactivity was found in the buccal ganglia. The fraction of this eluted with ^{3}H-ACh was equivalent to 21.5 $\pm$ 2.5% of the buccal radioactivity.

In the remaining five neurones an attempt was made to quantify the radioactivity in each lip nerve and the connectives. In no case was ^{3}H detected in either the ipsilateral, or contralateral medial lip nerves three hours after injection. The radioactivity in the other nerves and connectives was made up of choline (27.1%), betaine and phosphorylcholine (48.6%) and acetylcholine (24.3%). The highest proportion of ACh occurred in the contralateral lip nerves and connectives

where it was equivalent to 67 $\pm$ 9.1% of the total radioactivity compared with 22.8 $\pm$ 5% in the ipsilateral cerebro-buccal connective.

Figure 17.

Distribution of radioactivity in electropherograms of GSC extracts one hour after ^{3}H-choline injection.

The upper part of each figure represents tracings made of the electropherograms after they had been stained with iodine vapour. The histograms display the total cpm eluted from 5 mm strips of the electropherogram. Both experiments exhibit distinct peaks corresponding to acetylcholine (A) and choline (C).

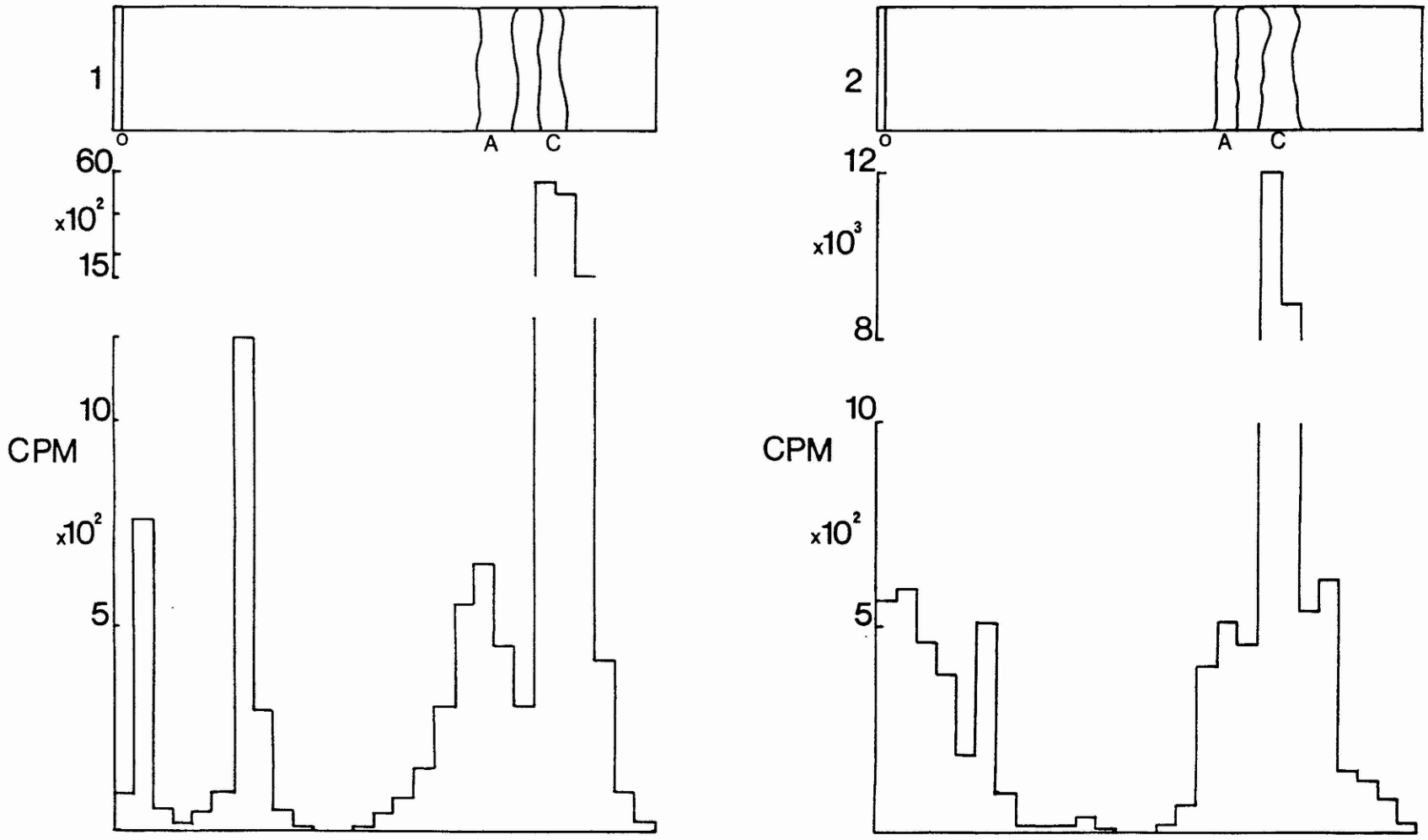
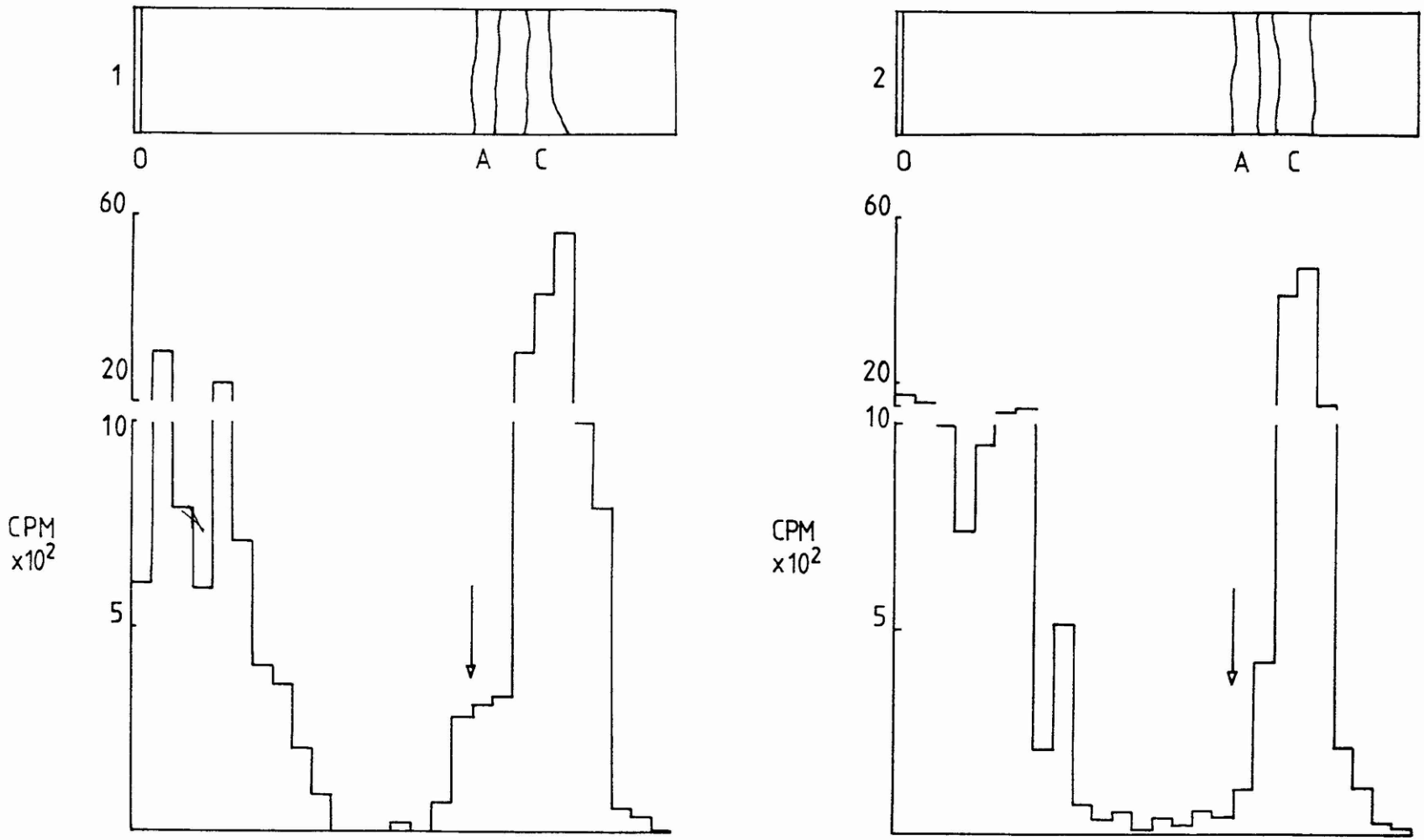

1
O
A
C
60
x10²
15
10
CPM
x10²
5
2
O
A
C
12
x10³
8
10
CPM
x10²
5

Figure 18.

Evidence that the radioactive peak eluting with acetylcholine after ^{3}H-choline injection into isolated GSC is ^{3}H-ACh.

1. One half of GSC extract subjected to electrophoresis exhibits a shoulder on the ^{3}H-choline peak which corresponds to the acetylcholine standard (A).

2. The other half of the extract after heat (40°) and alkali (1M KOH) treatment exhibits no ^{3}H-ACh shoulder.


1
O
A
C
60
20
10
5
CPM
x10²
2
O
A
C
60
20
10
5
CPM
x10²

Chapter 4

Intracellular injection of Radiochemicals: Discussion

4.1. Transmitter biosynthesis from exogenously derived precursors

Molluscan neurones possess the ability to accumulate amines and amino acids from their immediate environment (Carpenter et al.,1971; Pentreath and Cottrell, 1973; Osborne and Neuhoff, 1974; Eisenstadt et al., 1975; Osborne, 1975, 1976; Pentreath and Berry, 1978; Turner and Cottrell, 1978) as do their mammalian counterparts (eg. see Iversen, 1975, 1977). One function of these uptake mechanisms in both mammals and molluscs may be involved with transmitter inactivation (Cottrell, 1971b;Gerschenfeld, 1973; Iversen, 1975, 1977; Gerschenfeld et al., 1978) since, for example, both 5-HT and dopamine uptake sites are located at neuronal terminals (Bloom and Costa, 1971; Pentreath and Cottrell, 1973; Cuello and Iverson, 1973; Pentreath and Berry, 1978; Turner and Cottrell, 1978) and uptake inhibitors potentiate the effects of released 5-HT (Cottrell, 1971b;Gerschenfeld et al.,1978).

Molluscan neurones can also accumulate transmitter precursors such as 5-HTP (Carpenter et al.,1971; Pentreath and Cottrell, 1973; Turner and Cottrell, 1978) dopa, (Osborne, 1975; Turner and Cottrell, 1978) and choline (Schwartz et al.,1975; Osborne, 1976). Furthermore in identifiable neurones these sequestered precursors can be converted to 5-HT (Osborne, 1973) dopamine (Osborne, 1975) and ACh (Eisenstadt et al., 1975; Osborne, 1976). The uptake sites for the biogenic amine precursors 5-HTP and dopa are located solely on neuronal perikarya and proximal axons and the two amines are probably sequestered by the same mechanism (Turner and Cottrell, 1978). There are two distinct choline uptake mechanisms in Aplysia (Schwartz et al.,1975) and Helix (Osborne, 1976). The high affinity choline uptake mechanism (Km. 2-8 μM) in Aplysia is

preferentially located in the terminals of cholinergic neurones and not found in cell bodies (Eisenstadt et al., 1975) whereas the low affinity mechanism is more generally distributed throughout the nervous system (Schwartz et al.,1975). L-amino acids are sequestered by most neurones in the molluscan CNS and by several non neuronal components (Pentreath and Cottrell, 1973; Osborne, 1975; Weinreich and Weinreich, 1977; Pentreath and Berry, 1978; Turner and Cottrell, 1978).

Thus molluscan nervous tissue can sequester and metabolise a variety of substances. Consequently it is important that metabolites detected by chromatography or electrophoresis have been derived solely from the injected neurone and not from material which has leaked out, been sequestered and subsequently metabolised by other neurones or glia.

The data presented in this study (see Table 3.1.) and in other studies (Eisenstadt et al.,1973; Goldman and Schwartz, 1974) demonstrate that a large proportion of injected radioisotopes leak from the injected neurone. For example, up to 60% of the injected ^{3}H-tryptophan could be detected in the perfusate after 6 hours in Helix (Table 3.1.) and 70% in Aplysia (Eisenstadt et al.,1973). However, in this study there are several reasons for suggesting that only an insignificant portion, if any at all, of the intraneuronal ^{3}H associated with putative transmitters was derived from sequestered material.

The bath and perfusion system was designed that any radioactivity from injected neurones was quickly diluted and removed from the bath. The buccal ganglia - the region used to analyse ^{3}H in the endings of the GSC - were physically separate from the sites of injection except for the connectives. Thus radioactivity appearing in these ganglia could

only have arrived via the cerebro-buccal connectives and most likely from within the axons of the GSC. However uptake by a neurone which has axons in the connectives and endings in the buccal ganglia cannot be discounted but would seem unlikely as the distribution of radioactivity followed exactly the distribution of the GSC axons. Therefore any other neurone would presumeably have to be absolutely identical to the GSC in its gross morphology.

Autoradiographic studies have indicated that both injected transmitters and their precursors remain within the soma, axons and dendrites of the injected neurone (Pentreath and Cottrell, 1974; Pentreath and Berry, 1975; and see Figure 21) as do amino acids and sugars (Globus, Lux and Schubert, 1968; Thompson, Schwartz and Kandel, 1976). Although there is evidence that injected amino acids will exchange with glia cells (Globus, Lux and Schubert, 1973; and see Bradford, 1977) and that ^{3}H-fucose will cross electronic junctions (Thompson et al.,1976).

Finally there are several kinetic and biochemical studies which suggest that the synthesis is confined to the injected neurone. Although most of the choline accumulated in Helix and Aplysia neurones via the high affinity mechanism is readily converted to ACh, choline derived from the low affinity system is not (Schwartz et al., 1975; Osborne, 1976). In Helix the Km for the two processes is 1.7 µm and 100 µm (Osborne, 1976), thus at the choline concentration of the medium (50 µm) during the injection and in the immediately following phase the velocity of the low affinity mechanism is 2-3 x greater than high affinity mechanism (Osborne, 1976) and therefore accumulation via the former process will be preferred with a resultant reduced conversion to ACh.

Although tryptophan uptake appears to be a ubiquitous feature of Helix nervous tissue (Pentreath and Cottrell, 1973; Osborne 1973) the subsequent metabolism of tryptophan to 5-HT is restricted to 5-HT containing neurones (Osborne, 1973). Neurones which use dopamine or histamine as neurotransmitters also have selective mechanisms for the synthesis of dopamine from tyrosine (Osborne, Priggemeier and Neuhoff, 1975; Osborne, 1976) and histamine from histidine (Weinreich, 1976; Weinreich and Weinreich, 1977). 5-HTP can be accumulated by amine-containing neurones in Planorbis (Turner and Cottrell, 1975) but although AAD in non-5-HT-containing neurons may convert 5-HTP to 5-HT (see Emson and Fonnum, 1974), there is no evidence that accumulated 5-HTP is available for synthesis. Furthermore Pentreath and Cottrell (1973) have shown that the GSC's of Helix pomatia accumulate ^{3}H-5-HTP but the surrounding tissue does not. Therefore it is unlikely that 5-HT biosynthesis will take place in this region unless within the GSC itself.

Injection of large amounts of ^{3}H-5-HTP (see table 3.7.) and ^{3}H-choline (see table 3.15.) into the neuropil led to conversions to ^{3}H-5-HT and ^{3}H-ACh equivalent to only 15% and 9% of the corresponding values for injections into the GSC and MBC respectively. After ^{3}H-choline injection, the GSC s of Helix pomatia and Helix aspersa converted 2-3 x more of the substrate to ^{3}H-ACh than did the neuropil (Table 3.15.). Furthermore the data that cholinergic neurones were unable to convert tryptophan and 5-HTP to 5-HT and that the dopamine and serotonin neurones of Planorbis were unable to synthesise ACh from choline makes it unlikely that components other than the injected neurones are responsible for the sythetic processes.

Thus by a critical analysis of the experimental protocols and of

the evidence of other workers the experimental observations discussed in the following sections can be seen to be based on a reliable experimental technique. This suggests that the distribution and form of the radioactivity in the CNS following injection can be considered to be derived entirely from the injected neurone.

4.2. 5-Hydroxytryptamine synthesis in identifiable neurones

4.2. (1) Tryptophan hydroxylase

Using the intracellular injection of precursors the enzyme tryptophan hydroxylase appears to be selectively localised in Helix pomatia neurones which are believed to use 5-HT as a transmitter. The data are in good agreement with the only previous report using this technique for tryptophan injection in Aplysia (Eisenstadt et al., 1973) and also with the work of Osborne (1973) in Helix using tissue incubated in a medium containing ^{14}C-tryptophan.

The four different serotonin-containing neurones studied(two in Aplysia,(Eisenstadt et al.,1973) one in Helix and one in Planorbis, (this study),all converted different amounts of tryptophan to 5-HT. There are probably two main reasons for these differences. Firstly, there is a difference in volume between all four neurones (1 ml for Planorbis GSN to 14.6 nl for Aplysia GSC, (Weinreich et al.,1973)) and therefore the intracellular concentrations of the substrates will vary accordingly. Secondly there is a difference in neuronal activity in all the neurones, the RB cells in Aplysia which convert almost 10% of the tryptophan to 5-HT are the only spontaneously active neurones of the four (Koester, Mayeri, Liebeswar and Kandel, 1974). The GSN in Planorbis is more active (see Chapter 5) than either of the two GSC's which are normally silent (Eisenstadt et al., 1973; Cottrell and Macon, 1974) and

this reflects their transmitter synthesising capabilities (see table 3.3. and Eisenstadt et al., 1973). Increased neuronal activity is known to increase 5-HT turnover in the rat (Eccleston et al.,1970; Shields and Eccleston, 1972), to increase the fluorescence associated with nigral dopamine also in the rat (Lichtensteiger, Felix, Lienhart and Hefti, 1976) and to increase the dopamine fluorescence in an identified neurone of Planorbis (Lichtensteiger, Felix and Hefti, 1979).

The data obtained from the intracellular injection method cannot be used to derive the Michaelis constant (Km) for tryptophan hydroxylase as the total intracellular tryptophan concentrations, the concentrations of other substrates and thus the initial reaction rates are unknown. Amino acid levels have been measured in several invertebrate neurones and putative transmitter amino acids eg. glutamate, glycine and Gaba are present in high concentrations in individual neurones (Osborne, Sczcepaniak and Neuhoff, 1973; McBride, Shank, Freeman and Aprison, 1974; Zeman and Carpenter, 1975; Iliffe, McAdoo, Beyer and and Haber, 1977; and see Kehoe and Marder, 1976). Other amino acids are also present in molluscan neurones but not at such high concentrations (Osborne et al.,1973; Cottrell, 1974) and tryptophan concentrations are higher in 5-HT containing neurones than other cells (Osborne et al.,1973). However it would seem unlikely that the concentration of tryptophan in the Helix pomatia GSC's is very high as Osborne (1977) fails to report tryptophan in the GSC whereas at the same time he reports 70 µm Aspartate. Thus it seems likely that the level of endogenous tryptophan may be less than 10% of the injected ^{3}H-tryptophan at a concentration of 1 mM. At this concentration the activity of the hydroxylase can be estimated at 0.1 pmol/cell/hr after 1 hr but falls off to 0.03 pmols/cell/hr at 4,6 and 10 hours, assuming

complete conversion of 5-HTP to 5-HT. This assumption is probably quite valid as the activity of AAD in the GSC's with 5-HTP as a substrate is probably 15-20 pmols/cell/hr (data extrapolated from the results of Hanley et al.,(1974) using dopa as substrate and compared to corresponding data obtained in Helix aspersa GSC's by Emson and Fonnum (1974) using 5-HTP as substrate) and may be as great as 60 pmols /cell/hr (Cottrell and Powell, 1971). This supports the observation that tryptophan hydroxylation is the rate limiting step in the biosynthesis of 5-HT (see also Grahame-Smith, 1971; Carlsson, 1974). Further support for this hypothesis comes from the failure in these experiments, except at very high concentrations of tryptophan, to detect 5-HTP in the GSC. One reason may have been that the level was below the sensitivity of the extraction and assay procedure, or that as electrophoresis was followed by chromatography to separate tryptophan from 5-HTP (see Materials and Methods) there may be more chance of breakdown or loss of the products. The findings are rather surprising as both 5-HTP and 5-HT can be detected in Helix nervous tissue after incubation with tryptophan (Osborne, 1973; Osborne and Heuhoff, 1974) and a small portion of radioactivity (5%) is recoverable as ^{3}H-5-HTP after ^{3}H-tryptophan injection into Aplysia RB neurones (Goldman and Schwartz, 1974). In the Helix studies the 5-HTP levels in individual GSC's are very low after incubation with tryptophan (Osborne, 1973) but approximately two thirds of the 5-HT levels in whole tissue (Osborne and Neuhoff, 1974). It may, however, Be difficult to extrapolate the findings in whole tissue to individual neurones as there are several unknown parameters. For example tryptophan uptake is a general feature of molluscan tissue (Pentreath and Cottrell, 1973);

and tryptophan can be hydroxylated non enzymatically and by other hydroxylating enzymes (Renson, Weissbach and Udenfriend, 1962), although this is not the case one non-amine containing neurone in Helix (Osborne, 1973). Glial cells in mammals are known to accumulate amines and have been postulated as sites of glutamine synthesis from glutamate(Bradford, 1977) therefore metabolism in this constituent of molluscan CNS cannot be excluded. Finally one possible explanation may be that, like mammals, there are two tryptophan hydroxylating enzymes with different locations and different kinetic constants (Lovenberg et al.,1968; Ichyama, et al.,1970; Knapp and Mandell, 1973; Friedman et al.,1976) and therefore at different neuronal sites different reaction velocities are expressed.

The hydroxylation of tryptophan and the subsequent decarboxylation of the product to 5-HT did not appear to be a saturable process at the concentrations of tryptophan studied (see Figure 10) but the detection of 5-HTP after high tryptophan concentrations suggests that there may be a limiting component in the synthesis of 5-HT either at the decarboxylation step and/or at a step which might involve packaging of the 5-HT into storage vesicles.

The observation that tryptophan can be decarboxylated to tryptamine has not previously been reported in Helix neurones thus the demonstration of ^{3}H-tryptamine in the GSC, 10 hours after ^{3}H tryptophan injection is interesting. This pathway is not believed to exist in mammals under normal physiological conditions (Eccleston et al., 1966) but can be demonstrated after inhibition of tryptophan hydroxylase and monoamine oxidase (Axelrod and Saavedra, 1973). It is surprising, therefore, that ^{3}H-tryptamine should be detected in Helix without

probably only 50% of the true conversion rate as AAD will not metabolise D - 5-HTP (Goldman and Schwartz, 1974). The conversion rate for Aplysia GSC s is very similar, 51% compared with 56%, both of which are greater than the corresponding conversion (37%) in Aplysia RB cells (Goldman and Schwartz, 1974). However caution should be used when extrapolating enzymic activity data from conversion rates to give an indication of a neurones synthesising capability as many of the parameters involved in the enzymic conversion are not measurable in these experiments (see previous section(4.2.) for discussion of initial rates and substrate concentrations). One factor which may complicate the data is the observation that the process appeared to saturate at concentrations of substrate greater than 4 mM (see Figure 12). A similar saturation occurs in Aplysia GSC s for 5-HT synthesis at 5-HTP concentrations greater than 3 mM (Goldman and Schwartz, 1974) and for ACh synthesis at choline concentrations greater than 5 mM (Eisenstadt et al., 1973) in cholinergic neurones. It has been suggested that the saturation in Aplysia GSC s may be due to the unavailability of storage sites for the product (Goldman and Schwartz, 1974) and this may be the case in Helix. Transmitter packaging may limit the synthesis of 5-HT in Planorbis neurones as there is a large difference in the amount of 5-HTP converted to 5-HT in the two different neurone-types studied. The serotonin neurone converts almost twice as much 5-HTP to 5-HT as does the dopamine neurone (Table 3.9.). As there is no evidence for more than one decarboxylase in Helix nervous tissue (Emson and Fonnum, 1974) and as the two neurones studied are approximately the same size and exhibit similar neuronal activity (personal observations) the difference in conversion could be explained by lack

of storage sites in the GDC causing the free 5-HT to be metabolised. It is known in Aplysia that all neurones possess a mechanism for the conjugation of free 5-HT to a hexuronide (Goldman and Schwartz, 1971). Other factors may be responsible for the differences observed; for example, different unmeasured cofactorsconcentrations may be present in each neurone and although there is no direct evidence for two decarboxylases the ratio of 5-HTPD : dopa D activity does vary from neurone to neurone (Emson and Fonnum, 1974 and c.f. Sims and Bloom, 1972, 1973; Sims, 1974). Consequently the AAD within the dopamine neurone may have a low 5-HTPD activity compared to the AAD in the serotonin neurone. This suggestion however, may be unlikely as in both Aplysia (Weinreich et al., 1973)and Helix (Emson and Fonnum, 1974) dopa D activity is generally greater than 5-HTPD activity even in 5-HT-containing neurones.

At short time intervals 5-HT is the principle metabolite of 5-HTP in Helix pomatia GSC (Table 3.8.) however there is a gradual increase in the acid extractable material and in the radioactivity associated with the precipitate. At least part of the acid extractable fraction was probably 5-hydroxyindole acetic acid, a major metabolite of 5-HT in molluscs (Marsden, 1972; Osborne and Neuhoff, 1974) and in mammals (Greene and Grahame-Smith, 1975) but most of the acid extract was difficult to characterise as it remained near the origin during electrophoresis amd may therefore be associated with larger or uncharged molecules at this pH. In the non-5-HT-containing neurones 5-HTP accounted for only 20% of the intraneuronal material after 6 hours (c.f. 42% in the GSC's) and the remainder was unidentified indicating a more active catabolism of 5-HTP in non-amine containing neurones.

4.2. (3) Transport and release of newly synthesised ^{3}H-5-HT in Helix pomatia serotonin neurones.

Newly synthesised ^{3}H-5-HT is preferentially translocated into the axons of the GSC following intracellular injection of ^{3}H-tryptophan or ^{3}H-5-HTP. The enrichment of ^{3}H-5-HT in distal portions of the axons and the buccal ganglia after intrasomatic injection of ^{3}H-5-HTP has been also observed in Aplysia GSC's (Goldman and Schwartz, 1974; Goldman et al.,1976). A similar preferential transport mechanism of ^{3}H-ACh following ^{3}H-choline injection in cholinergic neurones (Koike et al.,1974) and for ^{3}H-glycoproteins following ^{3}H-fucose injection (Ambron et al.1974) has also been observed in Aplysia.

Biogenic amines in vertebrates are transported along axons (Dahlstrom, 1971; Dahlstrom et al., 1973; Fibiger, McGeer and Atmadja, 1973) in synaptic vesicles (see reviews by, Geffen and Livett, 1971; Wittaker and Zimmerman, 1974; Burnstock and Costa, 1975; Osborne, 1977). The transport of ^{3}H-5-HT in Aplysia serotonin neurones is by a mechanism associated with vesicles (Goldman and Schwartz, 1974; Goldman et al., 1976) and a similar mechanism probably occurs in other molluscs (Pentreath and Berry, 1978). The GSC's of Helix pomatia have been shown previously to contain dense-core synaptic vesicles (Pentreath et al.,1973) and injected ^{3}H-5-HT collocates with these vesicles observed by electron microscopic autoradiography (Pentreath, 1976). Furthermore Osborne (1978) has observed that 80% of injected ^{3}H-5-HT is associated with the particulate fraction after subcellular fractionation. Thus it is very likely that the newly synthesised ^{3}H-5-HT in the experiments reported here is associated with synaptic vesicles. Indirect evidence that ^{3}H-5-HT is transported by synaptic vesicles is derived from examination of the profiles of radioactivity in the cerebro-buccal connective (see Figure12).

If ^{3}H-5-HT was free in the cytoplasm the distribution of radioactivity would resemble a smoothly declining curve, commensurate with the kinetics of diffusion (Goldman et al.,1976). Small molecules, not believed to be transmitters, in Aplysia neurones have been shown to behave in exactly this manner (Koike et al.,1974 and see Goldman et al.,1976). The distribution of ^{3}H-5-HT in the Helix pomatia axons in peaks and troughs argues against simple diffusion as the mechanism of transport of this amine.

Calculation of the precise rate of ^{3}H-5-HT transport in these experiments is difficult as it is impossible to judge the exact location of the moving front of radioactivity. Rough estimates suggest the rate is probably around 2 mm.hr^{-1} which is quite close to similar estimates in Aplysia neurones.(70-120mm.day^{-1}, Goldman et al,1976; Goldberg et al., 1976). Transport rates in these studies are probably not accurate in light of the experiments of Goldberg and coworkers (1976). These authors demonstrated that if one branch of an axon was cut and the axoplasmic flow interrupted in Aplysia GSC s, an increased amount of ^{3}H-5-HT was transported in the uncut branch and also transported at an increased rate. The distribution of Helix pomatia GSC axons is much greater than that of Aplysia (Pentreath and Cottrell, 1974). Thus several branches will be cut when the CNS is dissected from the animal. Consequently both the rate and the amount of ^{3}H-5-HT transported in the Helix cerebro-buccal connectives may be far greater than in an in vivo situation. In contrast to this however, Goldberg and coworkers (1978) have observed transport of up to 130 mm/day in Aplysia GSC s using a stop-flow technique, so more refined studies would be required in Helix for accurate measurement of axoplasmic transport rates of ^{3}H-5-HT.

Stimulation induced release of a radioactive material which was probably ^{3}H-5-HT has already been demonstrated in Helix pomatia nervous tissue (Osborne and Neuhoff, 1974). The radioactive ^{3}H-5-HT had been derived from exogenous ^{3}H-tryptophan or ^{3}H-5-HTP but caution is required in interpretation of these results as accumulation and release of material can be effected via non neuronal tissue (Ascher, Glowinski, Tauc and Taxi, 1968; Carpenter et al., 1971).

The stimulation induced release of radioactive material in this study although insufficient to be analysed was probably ^{3}H-5-HT as this accounted for more than 80% of the buccal radioactivity. The low amount of ^{3}H-5-HT released is not surprising on two accounts, firstly, it is unlikely that the radioactive 5-HT accounts for all the 5-HT present in the GSC terminals and secondly it is probably unlikely that all uptake is blocked by imipramine the concentration used (10^{-5}M) (Osborne et al.,1974) calculated the ID_{50} value for imipramine to be 5.02×10^{-6}M, only 2 x lower than the concentration employed in my experiments). Interestingly, in a recent study (Gerschenfeld et al., 1978) 0.91 pmols of 5-HT were released by the Aplysia GSC s after 1 hour stimulation. This value is some 7 x more than the corresponding values calculated in this study but the points discussed above could easily account for the differences.

4.2. (4) Specificity of synthetic pathways for biogenic amines in molluscs

Tryptophan is generally believed to be the circulating precursor of 5-HT in mammals (see Green and Graheme-Smith, 1975). The situation in molluscs may be different as both tryptophan and 5-HTP are present in blood (Osborne, 1972b) and molluscan nervous tissue possesses an uptake mechanism for both substances (Eisenstadt et al.,

1973; Pentreath and Cottrell, 1973). Since tryptophan uptake may be a general property of Helix nervous tissue (Pentreath and Cottrell, 1973) and AAD is ubiquitously distributed in Aplysia neurones (Weinreich et al.,1973) some investigators have suggested that the presence of tryptophan hydroxylase is the unique distinguishing feature of a serotonergic neurone (Eisenstadt et al.1973; Goldman and Schwartz, 1977). Similarly tyrosine hydroxylase and histidine decarboxylase may be the distinguishing characteristics of dopaminergic and histaminergic neurones (Osborne, 1975; Weinreich, 1975). Alternatively the storage and transport of particular transmitter may also be specific to particular neurones.(Goldman and Schwartz, 1974).

At present it is unclear in Helix whether 5-HTP or tryptophan (or perhaps both?) is the blood-borne precursor of 5-HT. The more specific distribution of the synthetic enzymes (Emson and Fonnum, 1974; this study), suggest that either could fulfil the role. However, the observation by Turner and Cottrell, (1978) that both dopa and 5-HTP are sequested by the same mechanism in aminergic neurones in Planorbis suggests that uptake of 5-HTP alone may not be specific enough to distinguish between serotonergic and dopaminergic neurones (assuming that the sequested 5-HTP and dopa are equally available to the intracellular AAD). One interesting observation in this study was the reduced conversion of 5-HTP to 5-HT in a dopamine neurone compared to a serotonin neurone. This suggests that there is perhaps an enzymic or storage specificity for the "correct" substrate within the two neurones and that the dopamine neurone does not synthesise or store 5-HT as efficiently as is done in serotonin neurones.

Thus the overall specificity of pathways in molluscs are most certainly a combination of uptake, synthesis, storage and transport.

4.3. ^{3}H-Acetylcholine synthesis in identifiable neurones

4.3. (1) Choline acetyltransferase

Choline acetyltransferase is a soluble cytoplasmic enzyme which has a heterogeneous distribution within cholinergic neurones (see Hebb, 1972). In molluscs ChAT has been detected in extracts of the somata of Aplysia and Helix neurones (Giller and Schwartz, 1968, 1971a; McCaman and Dewhurst, 1970; Emson and Fonnum, 1974) and also in the dendrites and nerve endings where it may be associated with the high affinity uptake mechanism for choline (Eisenstadt et al.,1975).

Intracellular injection of ^{3}H-choline into Aplysia neurones has shown that ChAT is present in the somata of identified cholinergic neurones and therefore the conversion of choline to acetylcholine can be used as a marker for these cells (Eisenstadt et al., 1973; Koike et al., 1974; Schwartz, 1974; Eisenstadt and Schwartz, 1975). The results of this study support the observationsmade on Aplysia cholinergic neurones. Helix pomatia cholinergic neurones will convert up to almost 70% of injected ^{3}H-choline to ^{3}H-ACh within an hour of injection which is very similar to the values for Aplysia cholinergic neurones (Eisenstadt et al.,1973; Koike et al.,1974; Eisenstadt and Schwartz, 1975). At the concentrations studied in Helix neurones the conversion process did not appear to saturate (Figure 14) whereas in Aplysia, between 5 and 10 mM the conversion saturated and beyond 10 mM was inhibited (Eisenstadt et al.1973). It was impossible to reach such high concentrations in the much smaller Helix neurones as the size limited the actual volumes of solution which could be injected. In Helix neurones slightly more radioactivity is oxidised to betaine, converted to phosphorylcholine or incorporated into lipid than in Aplysia ; this may be due to the

slightly lower conversion to ACh in the Helix neurones.leaving more choline available for other metabolic pathways.

ChAT is not present in the Planorbis serotonin neurone or dopamine neurone but the GSC's of Helix aspersa possess the ability to convert injected ^{3}H-choline to ^{3}H-ACh, confirming the observations of Emson and Fonnum (1974) and Hanley et al.,(1974).

Both Helix GSC s converted up to 15% of the injected choline to ACh in one hour. Interestingly this is less than one quarter of the conversion in cholinergic neurones in both Helix and Aplysia and also less than one quarter of the conversion of 5-HTP to 5-HT in the same serotonin neurones. The ^{3}H-choline not acetylated was metabolised in a similar manner to non-acetylated choline in cholinergic neurones but the proportion of ^{3}H associated with each fraction was different. In the Helix pomatia GSC s 42% of the radioactivity was recoverable as ^{3}H-choline after one hour compared to 8% in the cholinergic buccal neurone (Table 3.14.) and more ^{3}H-choline was oxidased to betaine in the serotonin neurone. The proportion of radioactivity incorporated into lipid, and non extractable material and the proportion phosphorylated was similar in the two neuronal types and also to Aplysia neurones (Eisenstadt and Schwartz, 1975) and probably reflects a general presence of choline kinase and phosphocholine phosphatase, enzymes which are present in most Aplysia neurones (Dewhurst, 1972; McCaman and McCaman, 1976).

4.3. (2) Transport and release of ^{3}H-acetylcholine in Helix serotonin neurones

^{3}H-Acetylcholine, synthesised in the cell bodies of Helix pomatia GSC s is transported from the somata within the axons of the neurones. Distal regions of the axon, and especially the buccal ganglia,

are progressively enriched with ^{3}H-ACh compared to other tritiated metabolites. The movement of ^{3}H-ACh appears to be solely connected with axons of the GSC and no radioactivity could be detected in nerves which did not contain GSC' axons. Thus Helix pomatia GSC's possess the ability to synthesise and transport ^{3}H-ACh to the nerve endings.

The transport mechanism for ^{3}H-ACh in the GSC axons within the cerebro-buccal connectives may be by a different mechanism than that for ^{3}H-5-HT synthesised from ^{3}H-5-HTP (compare Figures 12 and 15). The absence of moving fronts or waves except possibly in the ten hour experiment may suggest that ^{3}H-ACh is not packaged and transported like other transmitters in molluscs (see 4.3. (2) for discussion). However as acetylcholine is known to exist in brain in several compartments (see Marchbanks, 1977) and is only released from a cytoplasmic and not vesicular pool (Birks, 1974; also see Marchbanks, 1977) its transmitter function might not necessarily depend on its location and transport within vesicles. Furthermore, ^{3}H-ACh remains in the soluble fraction in axons of Aplysia neurones (Treistman and Schwartz, 1977). In addition Tauc and coworkers have suggested that ACh may be released from an extra vesicular compartment in one identified cholinergic neurone in Aplysia (Tauc et al., 1974; Tauc, 1977).

Thus sequestering and transport of ^{3}H-ACh by vesicles, although observed to occur in mammals (see Katz, 1966, 1969) and molluscs (Koike et al., 1972; Eisenstadt and Schwartz, 1975), need not be the only route for a transmitter to reach nerve terminals (see also Chapters 1-7 in Cottrell and Usherwood, 1977).

Stimulation of the GSC's after ^{3}H-choline injection failed to produce detectable levels of ^{3}H-ACh in the perfusate from the buccal ganglia. There was, however, an increase in the amount of ^{3}H-choline in the

perfusing fluid. In a previous study, Koike et al.,(1974) attempted to demonstrate a stimulation dependent release of ^{3}H-ACh from the terminals of an identified cholinergic neurone in Aplysia. In spite of using both eserine and hemicholinium-3 to prevent breakdown of ACh and reuptake of choline, only ^{3}H-choline could be detected and these authors concluded that the cholinesterase activity was too potent or too selectively localised to be inhibited. Thus inability to detect ^{3}H-ACh released by stimulation within the Helix serotonin neurones need not exclude it from a transmitter role within the GSC's.

4.3. (3) Isolated neurone experiments

The isolated neurone preparation used in this study demonstrated that the soma of the GSC's contain choline acetyltransferase activity. Invertebrate neurones are devoid of axosomatic synapses (Rosenbluth, 1963; Coggleshall, 1967) therefore transmitter synthesis in this region is unlikely to have arisen from material sequestered by nerve terminals. Nevertheless molluscan neurones are encapsulated with a sheath of neuroglia which invaginate the somata (see Figure 5, Pentreath et al., 1973) and contamination of dissected neurones by small adherent neurones has been noted (see Figure 1.c in Giller and Schwartz, 1971a). However it has been generally assumed that glial cells do not contain transmitter synthesising enzymes or transmitters (Hebb, 1961, 1972; McCaman and McCaman, 1976; but see Dennis and Miledi (1974) who have demonstrated ACh release from Schwann cells).

Completely isolated molluscan neurones retain their electrical properties in culture (Kostenko, Geletyuk and Verprintsev, 1974; Ger and Zeimal, 1976) and it is unlikely that the biochemical properties of the GSC will be changed in the isolated experiments. One interesting observation is that both isolated and intact preparations convert equal

amounts of ^{3}H-choline to ^{3}H-ACh which suggests that the biochemical properties of the enzyme have not changed in these experiments.

4.4. Dale's Principle : Does it apply to the Helix metacerebral neurones?

In a recent review Burnstock (1976) has suggested that there is good evidence for a re-examination of the concept that each neurone synthesises and releases only one neurotransmitter.

For some time it has been known that cells in the vertebrate gastrointestinal system can store a peptide hormone and a biologically active amine (see Thompson, 1965; and see Coupland and Fujita, 1976). More recent studies have shown that some sympathetic ganglia neurones contain in addition to noradrenaline, the peptide somatostatin (Hockfelt, Elfvin, Elde, Schultzberg, Goldstein and Luft, 1977). A second peptide, substance P (S-P), has also been identified in 5-HT containing neurones in rat brain (Hockfelt, Ljungdahl, Steinbusch, Verhofstad, Nilsson, Brodin, Pernow and Goldstein, 1978). Both peptides, somatostatin and S-P, may be transmitters in the CNS of vertebrates (see review by Otsuka and Takahashi, 1977; Wolstencroft, 1978).

There is also compelling evidence that adenosine triphosphate (ATP) a putative transmitter in the mammalian peripheral nervous system, (see Burnstock, 1975) is stored and released with noradrenaline in adrenergic neurones (Geffen and Livett, 1971; Su, Bevan and Burnstock, 1971; and see Burnstock, 1975, 1976). Thus in adult vertebrates there is good evidence for the coexistence of more than one neurally active substance within one neurone.

Interesting observations have been made on embryonic mammalian nervous tissue grown in vivo and in vitro which suggest that the final

transmitter characteristics of a particular nerve cell can be varied by changing its environment.

For example, Le Douarin and colleagues (1974, 1975) have shown that quail neural crest cells which are destined to become cholinergic became adrenergic when transplanted to adrenomedullary regions of the chick. Conversely neural crest cells destined for adrenomedullary cells do not differentiate to adrenergic neurones when transplanted in regions which normally give rise to the cholinergic enteric ganglion.

In vitro experiments with cultured embryonic neuronal tissue have shown that sympathetic neurones can develop cholinergic characteristics (O'Lague, Obata, Claude, Furshpan and Potter, 1974) and can make cholinergic synapses with other neurones (O'Lague et al., 1974; Ko, Burton, Johnson and Bunge, 1976) with skeletal myotubes (Nurse and O'Lague, 1975) and with cardiac myocytes (Furshpan, MacLeish, O'Lague and Potter, 1976). Some embryonic sympathetic neurones in culture can release both ACh and noradrenaline from different endings of the same neurone (Furshpan et al., 1976). The development of cholinergic or dual function characteristics in cultured neurones is well correlated with the development of the enzymes responsible for synthesis of both transmitters and the particular vesicle cytochemistry associated with noradrenaline and acetylcholine. Choline acetylase, tyrosine hydroxylase and dopa decarboxylase activity has been detected with the same neurone or identical group of neurones (Johnson, Ross, Meyers, Ross, Bunge, Wakshull and Burton, 1976; Green and Reine 1977; Ross, Johnson and Bunge, 1977). Furthermore the development of each enzyme in culture has a different time course and is age dependent (Johnson et al.,1976; Ross et al.,1977). Cultured sympathetic neurones contain both dense cored

noradrenergic vesicles and clear cholinergic vesicles which correlate with the development of enzymatic and electrophysiological characteristics (Landis, 1976; Ross et al.,1977). However only one type of vesicle is seen at each ending (Landis, 1976; Johnson et al.,1976) although one particular neurone may have two functionally and anatomically distinct endings (Furshpan et al.,1976; Johnson et al.1976; Landis et al., 1976). Therefore in culture individual vertebrate neurones can synthesise and release more than one neurotransmitter.

The development of sensitive microassay techniques has led to a number of publications reporting the coexistence of several putative neurotransmitters within single molluscan neurones. (Kerkut et al., 1966; 1967; Giller and Schwartz, 1971; Weinreich et al.,1973; Brownstein et al.,1974; Cottrell, 1974; Hanley and Cottrell, 1974; Weinreich, 1975, 1978; Saavedra, 1978 and see review by Kehoe and Marder, 1976).

The coexistence of several putative transmitters within one neurone does not necessarily imply that each one is released in a functionally significant manner or location. Furthermore there are several well established criteria to be fulfilled before a neuroactive substance can be considered as a neurotransmitter. At present, the situation in molluscs, especially Aplysia, while interesting, has not been shown to have a physiological significance and several pieces of evidence indicate that individual neurones probably use only one transmitter substance. For instance in neurones where a transmitter function is suspected for one particular amine that substance has been found to be present in much higher concentrations than the other amines or amino acids present eg. 5-HT in the metacerebral cells of Aplysia (Brownstein et al.1974) and Helix (Osborne, 1977); acetylcholine in identifiable

cholinergic neurones of Aplysia (Brownstein et al., 1974); histamine in identifiable histaminergic neurones in Aplysia (Weinreich, 1975) (and see Table 1 from Kehoe and Marder, 1976). In addition, although some neurones contain enzymes in the synthetic pathways of different transmitters and can synthesise different transmitters from their precursors (see Table 1, Kehoe and Marder, 1976) only the transmitter that has shown to be physiologically significant, e.g. 5-HT in Aplysia metacerebral neurones, is packaged and transported (Goldman and Schwartz, 1974). Finally the presence of some amines in Aplysia neurones are probably due to an uptake mechanism rather than de novo synthesis within the cell as, for example, although histamine has been detected in some Aplysia neurones the enzyme responsible for its synthesis, histidine decarboxylase is lacking (Weinreich and Yu, 1977, Weinreich, 1978).

In no case in Aplysia has more than the substance been shown to be released at a functional synapse. However in Helix the evidence is quite compelling that both 5-HT and ACh together mediate the connection between the GSC and the medial buccal neurone (Cottrell, 1976, 1977). In vitro biochemical studies have demonstrated that the synthesising enzymes AAD and ChAT (Osborne, 1973; Emson and Fonnum, 1974; Hanley et al., 1974) and also ACh and 5-HT (Cottrell and Osborne, 1971; Osborne and Cottrell, 1971; Hanley and Cottrell, 1974) are present in the cytoplasm of the GSC's. Further more electronmicroscope studies have shown the presence of two distinct vesicle types within axons and terminals of the GSC (Pentreath, 1976).

The biochemical evidence for the coexistance of ACh and 5-HT within the Helix GSC s has been challenged by Osborne (1977). However several mathematical inconsistencies in his paper suggest that his conclusions should be treated with caution. For instance, the amount of 5-HT per

GSC calculated from his figures (Table 1, Osborne, 1977) is equivalent to 80 pg per neurone. This value is more than an order of magnitude less than the amount of 5-HT (1.1ng) in these neurones reported previously by this author using identical techniques (Osborne, 1972 and see Cottrell, 1977). Furthermore the concentrations of two other amines in the GSC, histamine and dopamine (2×10^{-8}M and 8×10^{-9}M) are equivalent to less than 3 fg per neurone, which is considerably below the sensitivity the author claims to achieve (100 pg and 50 pg). Thus some of the data in this publication may be unreliable.

It has been suggested that trace amount of amines or amino acids in molluscan neurones can arise either by contamination or as a result of biological "noise" (see Giacobini, 1978). The nuclei of some Aplysia neurones contain 200,000 times as much DNA as Aplysia sperm cells (Lasek and Dower, 1971). Aplysia giant neurones may have resulted therefore from fusion of smaller cells and consequently contain the genetic machinery for multiple transmitter synthesis. Since all neurones should contain the same DNA initially the differentiation of transmitter function may arise from either gene suppression or gene induction. Substrate induced synthesis of enzymes is well understood in microorganisms (West, Todd, Mason and Bruggen, 1966; Stadtman, 1970) and is also known to occur in animal cells (Stadtman, 1970). Thus high levels of particular substrates could possibly promote synthesis of enzymes in molluscan neurones as intracellular injection produces levels more than 10 times normal. Such a process is unlikely in molluscan neurones since gene expression and protein metabolism is normally very stable in Aplysia (see Wilson, 1978). The absence of ACh synthesis in aminergic neurones or 5-HT synthesis in cholinergic neurones reported in this study indicates that gene expression in Helix and Planorbis neurones is probably also stable.

The results of intracellular injection studies demonstrate that the GSC's of Helix aspersa and Helix pomatia can synthesise both 5-HT and ACh from their precursors. Furthermore both transmitters move from the soma of the GSC to the GSC terminals in the buccal ganglia where they may be released (this study and see Cottrell, 1977). Thus in light of the evidence from this study and the reports of others (Emson and Fonnum, 1974; Hanley, 1974; Hanley and Cottrell, 1976; Cottrell, 1976, 1977) it can be postulated that the GSC's of Helix synthesise, transport and possibly release two different transmitters.

The functional role of two transmitters in the Helix metacerebral neurones is as yet, unclear. It is tempting to speculate, however, that if both substances are released by the GSC their roles may not be identical. There is compelling evidence that ACh functions as a conventional transmitter in the molluscan central and peripheral nervous systems (see reviews by Gerschenfeld, 1973; Ascher and Kehoe, 1975; Kehoe and Marder, 1976, McCaman and McCaman, 1976). 5-HT probably functions likewise in the molluscan CNS (see Gerschenfeld, 1973; Gerschenfeld and Paupardin-Tritsch, 1974b; Cottrell, 1977). There is evidence that 5-HT may have an indirect modulatory role in molluscs. For example, 5-HT potentiates muscle contraction and neuronal excitability in molluscs (Twarog, 1967; Hill, 1970; Salanki, 1970; Weiss, Cohen and Kupferman, 1975, 1978). Stimulation of the Aplysia GSC potentiates contraction of buccal muscles evoked by cholinergic motoneurones both through a direct effect on the motoneurones and at the neuromuscular junction (Weiss et al., 1975, 1978). Interestingly, other metacerebral serotonin neurones have been postulated as modulators of buccal motoneurones and of buccal musculature (Pentreath, 1973; Berry, Cottrell and Pentreath, 1974; Berry and Pentreath, 1976a; Gillette and Davis, 1977).

5-HT has also been postulated as a modulator of heterosynaptic facilitation and inhibition (Shimahara and Tauc, 1975; Tremblay, Woodson, Schlapfer and Barondes, 1976; Woodson, Tremblay, Schlapfer and Barondes, 1976) and may exert these effects by inducing the synthesis of cyclic AMP (Shimahara and Tauc, 1977; Weiss, Schonberg, Mandelbaum and Kupferman, 1978). Finally 5-HT levels in the CNS of Helix pomatia are dependent on variations in activity and on the season (Hiripi and Salanki, 1973). Thus in addition to a transmitter role in the molluscan CNS, 5-HT may subserve a hormonal or neuromodulatory function. Consequently, the two transmitters postulated to be present in the Helix GSC s may fulfil different functions and be present in different amounts throughout the year.

PART 2

CHAPTER 5. Studies on an identifiable 5-hydroxytryptamine-containing neurone in the right pedal ganglion of Planorbis corneus.

5.1. Introduction.

The evidence that 5-HT functions as a neurotransmitter in molluscs and the roles of identifiable 5-HT-containing neurones has been discussed previously (see Chapter 1.2.). The first part of this thesis describes data which suggest that Helix pomatia serotonin neurones synthesise, transport and release both 5-HT and ACh consequently they may not be good models of serotonergic neurotransmission.

In addition to the persuasive biochemical evidence, there are certain electrophysiological observations which detract from the usefulness of the Helix and Aplysia serotonin neurone model.

For example, although the Helix GSC's make excitatory connections with three identifiable buccal neurones (Cottrell, 1971) and an inhibitory connection with an unidentifiable neurone (Cottrell et al., 1974; Cottrell, 1977) the data on all but the medial buccal cell (MBC) connection is preliminary. In order to observe epsp's in this neurone after hyperpolarisation, a non-physiological K^+ free medium is required to abolish anomalous rectification (Cottrell and Macon, 1974). Furthermore the majority of iontophoresis and epsp data published this far have come from studies made on the GSC-MBC connection (Cottrell, 1970; Cottrell and Macon, 1974). Although the evidence that the GSC-MBC connection is monosynaptic is compelling (see Chapter 1.2. (2) b) the posterior buccal cell (PBC) and anterior buccal cell (ABC) evidence is less so. The PBC epsps evoked by stimulation of the GSC's are considerably smaller than those in the MBC and can only be observed by hyper-

polarisation of the PBC membrane (Cottrell, 1971; Cottrell and Macon, 1974). The GSC-ABC connection exhibits a long delay (8-10s) before onset of the response and intense stimulation is often required (Cottrell, 1971). Discrete epsp's have never been observed and the response lasts up to 15s after GSC stimulation has ceased. (Cottrell, 1971).

Although Aplysia GSC's make synaptic connections with at least thirteen neurones in each ipsilateral buccal ganglion (Gerschenfeld and Paupardin-Tritsch, 1974b) only two buccal follower neurones can be easily and repeatedly identified. The Aplysia buccal cells also exhibit anomalous rectification so hindering analysis of excitatory responses which generally consisted of slow waves of depolarisation (Gerschenfeld and Paupardin-Tritsch 1974a,b). Unitary epsp's (usually less than 1 mV) are only occasionally observed and unitary ipsps have never been recorded (Gerschenfeld and Paupardin-Tritsch, 1974b). There is also evidence that the Aplysia GSC's, like the Helix GSC's contain choline acetyltransferase (McCaman and McCaman, 1976).

Thus, there is both biochemical and electrophysiological evidence for looking for models of serotonergic transmission in molluscs in addition to the serotonergic metacerebral neurones and their follower cells.

The central ganglia of Planorbis corneus contain 3µg 5-HT per g wet weight of tissue. (Marsden and Kerkut, 1970). Fluorescence histochemical studies have demonstrated the existence of several large identifiable neuronal somata which contain 5-HT (Marsden and Kerkut, 1970; Turner, 1973; Berry and Pentreath, 1976). The metacerebral 5-HT neurones of this species have already been studied but no direct monosynaptic

connections with follower neurones have been observed (Berry et al., 1974; Berry and Pentreath, 1976). One of the largest neurones in the Planorbis CNS contains dopamine (Marsden and Kerkut, 1970 ;Powell and Cottrell, 1974) and makes monosynaptic connections with other neurones in the brain (Berry and Cottrell, 1974). Consequently this neurone has been used as a model for studying the mechanisms of dopamine-mediated synaptic transmission (Cottrell, Berry and Macon, 1974).

Another large identifiable neurone in a bilaterally symmetrical position to the dopamine-containing neurone (GDC) exhibits 5-HT fluorescence (Marsden and Kerkut, 1970; Turner, 1973; Osborne et al., 1975) and probably contains 5-HT as determined by bioassay (Purchon, 1976). This giant serotonin-containing neurone of Planorbis (GSN) will synthesise ^{3}H-5-HT from ^{3}H-5-HTP but not ^{3}H-ACh from ^{3}H-choline (see Chapter 3, tables 3.9. and 3.15.). Thus a study of the anatomical, biochemical, electrophysiological and pharmacological properties of this neurone will provide more information concerning the properties of identifiable 5-HT-containing neurones in molluscs.

Plan of investigation

The cytoarchitecture of the giant serotonin-containing neurone will be studied by intracellular injection of marker substances followed by examination at the light microscope level. Electrophysiological techniques will be used to confirm some of the neuroanatomical observations and also to look for connections between the GSN and neurones which have been identified in regions containing terminals of the GSN. Finally a study of the receptors on the soma of the GSN will be made and the results compared to the properties of other identifiable neurones.

5.2. Materials and Methods

Specimens of Planorbis corneus were obtained from T. Gerrard & Co., Littlehampton, Sussex, kept in large tanks of areated tap water and fed on pond weed.

5.2. (1) Dissection

The shell of the snail was cut anterio-posteriorly and removed. The animal was then pinned dorsal side upper-most to the base of a wax filled dish and the skin was then slit in the dorsal midline from the mantle to the tip of the head exposing the buccal mass. The buccal mass was then pulled forward and pinned to the dish revealing the ring of ganglia surrounding the oesophagus. The circumoesophageal nerve was freed by cutting the oesophagus posteriorly and pulling it through the ring and also by cutting all the nerves running from the ganglia. As long as a length of nerve as possible was left attached to the ganglia and great care was taken to avoid damaging these nerves which were subsequently to be stimulated.

The central ganglia and attached nerves was then removed from the animal and placed in a small Perspex bath (vol 0.5 ml) with a plastic base, pinned ventral side uppermost and continually perfused with physiological solution (Berry, 1972). The neuronal somata could be seen through the transparent connective tissue sheath encapsulating the ganglia and when the pedal ganglia were stretched in an anterior direction the GSN was clearly visible in the right pedal ganglion. The GSN could be easily distinguished from other neurones in the ganglion by its size (Ca 150 µm) and its position. Before an electrode could be inserted into any of the neurones the connective tissue sheath was gently removed with fine forceps.

In some of the experiments to investigate whether the GSN made

connections with neurones on the dorsal surface of the brain the left pedal-pleural connective was cut and the pleural, parietal and visceral ganglia were turned over to expose their dorsal surfaces.

In some experiments high-calcium (60 mM) and nominally zero calcium plus high magnesium (0mMCa^{++} + 27mM Mg^{++}) solutions were used to perfuse the ganglia. No compensation was made for the increase in chloride concentrations or for the increase in osmolarity.

5.2. (2) Neuroanatomy

The morphology of the GSN was studied using three intracellular staining techniques.

(i) Cobalt chloride ($CoCl_2$)

Somata were filled with cobalt using modifications of the procedures of Pitman et al., (1972). Single barrel glass micropipettes were filled with a solution of 1M$CoCl_2$ and those electrodes with a resistance greater than 25 Mohm were discarded. The GSN was exposed as described earlier, impaled with the microelectrode and cobalt ejected as a cation. Current pulses of 10-50 nA, 1s duration at 0.7 Hz for ½-2hrs were passed via a bridge circuit (see Figure 19.). The ganglia were left for 2-18 hours at 4°C and then immersed for 5 minutes in 1% ammonium sulphide made up in normal Planorbis physiological solution. The tissue was then washed, fixed in Bouins fixative and dehydrated through a phosphate buffered ethanol series (pH 7.4). After clearing in methylbenzoate the ganglia were mounted, whole, in a depression slide and viewed under a binocular microscope.

Three experiments were carried out using the pressure injecting technique (see Chapter 2 for details). A small volume (0.3 nl) of 1 M $CoCl_2$ was injected into the GSN and the tissue prepared as above.

Neither method was effective enough to permit visualisation of fine branches in regions distant from the cell body.

(ii) Procion yellow M4RS

Procion yellow M4RS (ICI) was washed in ether and made up in distilled water to a 4% solution (w/v). The electrodes, pulse intensity, duration and frequency were identical to those described for cobalt injection but Procion yellow was ejected as anion.

After leaving at 4°C for 2-18 hrs the tissues were fixed in glutaraldehyde or Bouins fixatives, dehydrated through an ethanol series and cleared in methyl benzoate. The tissue was then embedded in paraffin wax and 10 µm sections cut and mounted on glass slides. After dewaxing in xylene, sections were mounted in liquid paraffin and viewed in a Leitz Ortholux microscope using a Leitz BG-12, 1.5 mm excitation filter in conjunction with a Leitz barrier filter K530.

(iii) ^{3}H-5HT and ^{3}H-5HTP

1.0 ml to 0.1 ml aliquots of ^{3}H-5HT (10.7 Ci$mmol^{-1}$) and ^{3}H-5HTP (7.0 Ci.$mmol^{-1}$) were evaporated to dryness and resuspended in 20 µl of distilled water. Small portions of the solution were transferred to the tips of single barrel glass micropipettes and the GSN was impaled in the normal way. Both radiochemicals were ejected as cations using the same protocol as described in (i) above. After injection, tissues were left as in (i) and (ii), fixed in glutaraldehyde and prepared for autoradiography as described in (iv) Two injections were carried out using the pressure method and the tissues were processed as for current injected tissues.

(iv) Light microscope autoradiography

After glutaraldehyde fixation for 2-24 hrs at 4°C, tissues were dehydrated through an ethanol series and embedded in wax via

tertiary-butyl-alcohol. 7 µm sections were cut and mounted on albumin coated glass slides. Sections were then dewaxed in xylene and re-hydrated through an ethanol series. Slides were coated with Kodak AR 10 stripping film, dried and stored in the dark at 4°C to expose for 10-14 days. Autoradiograms were developed in Kodak D19, for 12 minutes at room temperature and fixed in 30% W/V sodium thiosulphate in tap water. Films were washed in distilled water, trimmed, and mounted wet in Farrants Medium (Gurr) and the slides were then viewed in a Leitz Ortholux microscope.

(v) Photography

All photographs were taken with a Leitz Leica camera. For cobalt and autoradiography Ilford Pan F or FP4 film was used and Kodachrome X was used for Procion yellow fluorescence.

(vi) Fixatives

a) Cobalt injection and light microscope autoradiography

1% glutaraldehyde in 0.0225 Mcacodylate buffer (pH7.3) for 2-24 hrs at 4°C.

b) Procion yellow injection : Alcoholic Bouins Fixative, 80% ethanol (150 ml); 100% formalin (60 ml); glacial acetic acid (15 ml); solid picric acid (1g), for 2-24 hrs at room temperature.

5.2. (3) Electrophysiology

(i) Intracellular recording and stimulating : Conventional single or double-barrelled glass microelectrodes were pulled on a mechanical electrode puller. The intracellular recording electrodes were filled with filtered solutions of ether 0.5 M K_2SO_4 or 2.5 M KCl. These electrodes had resistances in the range 5 to 30 M ohms. A silver/silver chloride wire connected the recording barrel of the electrode, via a

screened lead, to a "cathode-follower" type amplifier. The output from the amplifier was displayed on a Tektronix Type 502A oscilliscope.

The second barrel of double-barrelled microelectrodes was connected to a "backing off" circuit (see Fig.19) which was used to pass current into the neurone in order to adjust the resting potential to any desired level. When single barrelled electrodes were used for recording a "bridge circuit" (Figure 19) was incorporated into the circuit permitting simultaneous recording and passage of current. Square wave pulses could also be passed into the neurone by switching the bridge input of the second barrel to a Tektronix Type 161 stimulator.

In the experiments designed to see if the GSN made monosynaptic connections the following procedure was followed. The GSN was impaled with a double-barrelled, K_2SO_4 filled, microelectrode and hyperpolarised to prevent spontaneous firing. A second identical microelectrode was used to impale possible "follower" neurones which were left at their resting potential. A depolarising pulse was then passed into the GSN initiating a burst of action potentials and the membrane potential of the "follower" neurones observed to see if an p.s.p.'s had been elicited. The procedure was repeated as the "follower" neurone was hyperpolarised in - 5 mV stages, returned to its resting level and depolarised in + 5 MV stages. The reverse procedure was also followed to see if the "follower" neurones made monosynaptic connections with the GSN.

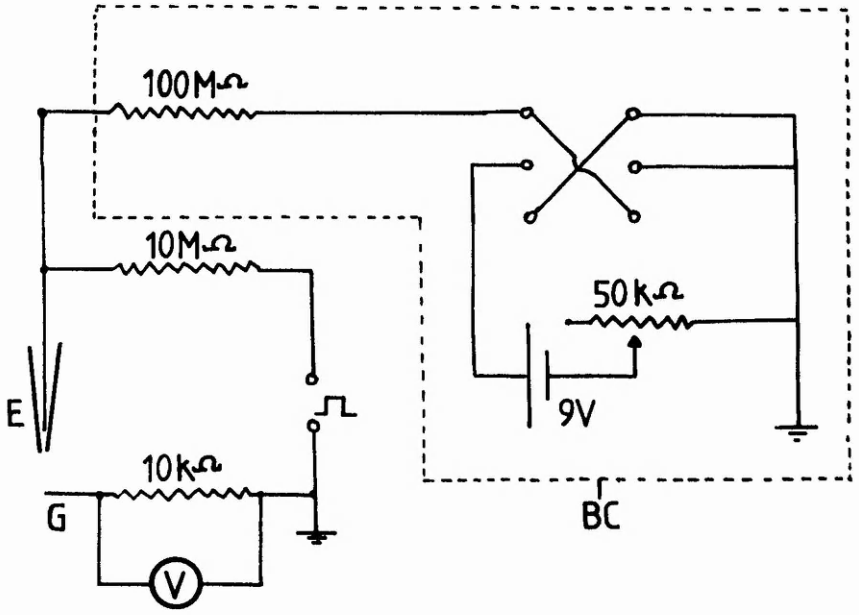
Initially the larger neurones on the ventral surface were examined with special emphasis on those situated in the visceral and parietal ganglia. Eventually all the large neurones and most of the smaller neurones on the ventral surface were examined. Neurones on the dorsal surface and in the cerebral ganglia were exposed after the left pedal-

Figure 19. Circuit diagrams of iontophoresis and bridge circuits.

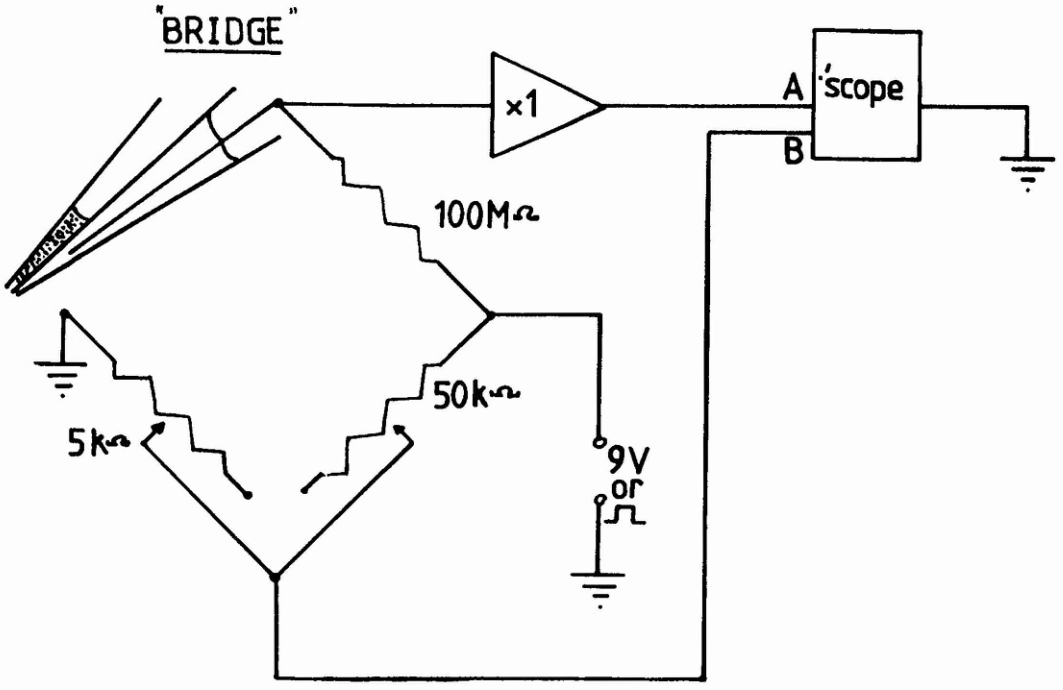
1. Iontophoresis. The iontophoresis ejecting current circuit consisted to a Tektronix variable pulse generator and power supply connected in series with the micropipette (E) via a 10 M Ω resistor. A 9V braking or retaining circuit (BC) was connected in series with the pipette by a 100 Mohm resistor and in parallel with the ejecting circuit. The braking circuit consisted of a power supply (9V battery) a 50 Kohm variable resistor and a dipole reversing switch in order that the polarity of the retaining current could be changed. Ejection currents were measured as the voltage drop across a 10 Kohm resistor placed between the silver/silver chloride bath earth (G) and the equipment earth.

2. Bridge. A wheatstone bridge circuit was used for simultaneous recording a passage of current during pressure injection experiments. Since the GSC s were often silent after they had been impaled with a microelectrode it was often necessary to depolarise the neurone causing a burst of action potentials to confirm that the electrode was still within the cell. A conventional wheatstone bridge circuit with two variable resistors was used. Balancing the bridge by varying the resistors ensured that there was no potential difference between the output of the amplifier and the bridge circuit (recorded differentially between channels A and B on the oscilloscope), Thus current could be passed into the neurone without large voltage changes being recorded on the oscilloscope. This circuit was also used when single-barrel electrodes were used for recording or for injecting intracellular stains.

IONTOPHORESIS



"BRIDGE"



pleural connective was cut and the circumoesophageal ganglia, excluding the pedals, were turned over.

Over 500 penetrations of follower cells were made in about 35 preparations. Cell maps were constructed for each experiment so that the approximate location of the neurones could be seen.

Three experiments were performed to investigate whether stimulation of the GSN had any effects on the postsynaptic potentials elicited in visceral or parietal neurones by the giant dopamine cell of the left pedal ganglion. Double barrel microelectrodes were placed in the GSN, GDC and one of its follower neurones. A response was elicited in the follower neurone by stimulation of the GDN and designated the control response. Experimental responses were recorded when the GSN was stimulated before, during and after the control response and any changes from the control response were observed.

(ii) Extracellular recording and stimulating.

These experiments were made by a suction electrode constructed from polythene tubing tapered at one end and connected to a 10 ml syringe at the other. The tip of the electrode was interchangeable with similar pieces of varying diameter ensuring a tight fit when nerves of different thickness were sucked up for recording or stimulating. A fine silver/silver chloride wire made contact with sucked up solution in the electrode and an indifferent electrode of silver/silver chloride was placed in the bath. The electrode was connected to a capacity coupled preamplifier (x 2000, cut off frequencies 80 Hz and 10 Khz) and then to one channel of the oscilloscope. A switch enabled the same electrode to be used for nerve stimulation.

The stimulating circuit consisted of an R.f. isolation unit connected to Tektronix Type 160, 161, 162 modules for variable pulse generation.

The nerves were stimulated by passing square wave pulses the frequency, intensity and duration of which could be varied. In all cases the minimum voltage required to elicit a full response was used to avoid damaging nerves.

(iii) Iontophoresis

Single or double barrelled glass micropipettes were filled with the following drugs made up in distilled water to the concentrations shown ACh,1M; 5-HT, 0.15M; DA, 1.3M;L-glutamate,0.5M; GABA, 0.5M; histamine, 0.5M. Usually a braking current of 50 n amps was applied initially. This current was then gradually reduced until a response was observed and then immediately increased by 5 to 10 nA. The ejection currents (0.1 - 1.0s, 10 - 300 nA) were measured as the potential drop across a 10 K ohm resistor in series with the iontophoresis pipette (see Figure 19) and displayed on the oscilliscope along with the intracellular record. In some experiments histamine, GABA and 5-HT were made up in physiological solution in concentrations of 10^{-2}M and applied to the GSN from a glass micropipette which was attached to a Prior manipulator. The tip of the micropipette; manufactured in a similar manner to a micro-electrode, was broken until it was 15-25 μm in diameter to enable the drug to diffuse out freely. The pipette was lowered until it was just below the surface of the continually perfusing saline, ensuring that any drug which diffused out was washed away. The pipette was then lowered further under visual control to a position almost touching the cell surface from which the diffusing drugs were washed over the cell soma, the pipette was retracted immediately a response was observed.

Permanent records were made on a Brush Clevite 2-channel pen recorder (Gould Instruments) or by direct photography of the oscilloscope screen using a Nihon Kohoden camera and Kodak 35 mm photographic paper or film.

5.2. (4) Chemicals

In addition to the chemicals listed in Chapter 2 the following substances were used : γ-aminobutyric acid; ethylenglycol-bis (β aminoethylether) N,N^1 - tetraacetic acid (EGTA); glutamic acid hydrochloride; glutaraldehyde; histamine acid phosphate; (all BDH); tubocurarine chloride; (Burroughs Wellcome).

5.3. Results

The photomicrograph in Figure 20 shows the ventral aspect of a whole mount preparation of the CNS of Planorbis corneus. The accompanying diagram is a representative drawing of the CNS with some of the prominent features labelled. The GSN (arrowed in photomicrograph) is clearly visible in the right pedal ganglion as are the GDC and another large neurone in the left pedal ganglion. The GDC is one of the largest neurones in the brain, varying in diameter from 150 to 200 µm whereas the GSN is slightly smaller usually in the range of 100 to 150 µm. There is considerable variation in the size and the colour of the neurones in the brain making it reasonably easy to identify particular neurones (especially the larger ones) from preparation to preparation. Several nerves are clearly visible in the photomicrograph and, where appropriate, these are named later in the text.

5.3. (1) Neuroanatomy

(i) Cobalt Figure 21 is a montage of photographs taken from a whole mount preparation after the GSN had been injected with cobalt. The soma and proximal segment of the axon are the most densely stained and approximately 1.3 mm of the main axon, varying in diameter from 15 µm to 5 µm is clearly visible passing out of the right pedal ganglion and through the right pleural and parietal ganglia. As the course of the axon is not always in the same plane it is difficult to photograph this whole mount preparation and impossible to show the ganglia clearly at the magnification required to see the axon and its branches. However the general course of the main axon can be clearly seen running close to the edge and quite near the surface of the ganglion before it crosses the pleural-parietal connective and enters the parietal ganglion. The main axon bifurcates shortly after leaving the soma (solid arrow) and a fine

branch 1-3 μm in diameter with several smaller branches can also be distinguished (small arrows). A bifurcation can also be observed in the edge of the parietal ganglion (small arrows at bottom of photomicrograph).

Although five GSN's were injected with cobalt, this technique did not provide much information about the fine structure of the neurone. However it did indicate some of the gross anatomical features of the GSN and demonstrated that the axon followed a course to the visceral and parietal gnaglia.

(ii) Procion yellow. After injection of four GSN's with Procion yellow, examination of whole mount preparations of the CNS provided no more information than had been already obtained from cobalt injection. In two cases it was impossible to see any fluorescence other than that in the somata. However after sectioning the CNS and viewing the 10μm sections in a fluorescence microscope more details of the cyto-architecture were visible.

Figure 22a shows the GSN soma, axon hillock and proximal section of the axon (arrow). The nucleus (n) is large and comprises approximately 2/3 of the total soma volume. The GSN axon in this section is 25 μm in diameter when it leaves the soma, has an uneven appearance and can be followed for 70-100 μm. The somata of several uninjected neurones are clearly visible in this section; all are noticably smaller than the GSN.

Figure 22b is a section of the same neurone taken deeper into the neuropil of the right pedal ganglion. The axon is very convoluted in this section and can be seen to bend back on itself (large arrows). This may have been an artifact of the fixation or sectioning technique as such severe convolutions were not observed in other experiments.

This photomicrograph should be compared with Figure 22c taken from another injected neurone. This is a section through the neuropil (N) of the right pedal ganglion. Very few cell bodies are present and the section consists mainly of axons and (presumably) dendrites. The GSN axon passes in a plane almost 90° to the plane of section for almost 300 µm. The mean diameter of the axon in this section is approximately 10 µm and some convolutions are visible (large arrows). Several fine branches are clearly visible projecting into the neuropil (small arrows). Figure 22d shows more clearly the ramification of large branches to produce several fine dendrites. One small process less than 1 µm (arrowed) could be followed in subsequent sections across the pedal-pedal commisure and into the contralateral pedal ganglion.

(iii) Autoradiography. Injection of ^{3}H-5-HT or ^{3}H-5-HTP followed by light microscope autoradiography demonstrated that extensive ramifications of the GSN axon were present in the right pedal ganglion neuropil (see Figure 23A). In addition to the branch which had been observed projecting to the contralateral pedal ganglion a second fine branch left the ipsilateral pedal ganglion via the superior pedal nerve (see Bullock and Horridge, 1965 and Elo, 1938 for nomenclature of nerves). The main axon approximately 10-15 µm in diameter was clearly visible in all the injected neurones and Figure 23B and C shows the axon from two different injected neurones. In 23B the main branch has passed out of the pedal ganglion and is easily located within the right pleural ganglion. Several small branches are clearly visible projecting into the neuropil of the pleural ganglion. Figure 23C is a section of the neuropil of the pedal ganglion where the main axon is seen projecting towards the pedal-pleural connective. Silver grains can also be observed over a cut axon in this figure (double arrows). This nerve could be followed

in subsequent sections and was either a right parietal nerve (N. pallialis) or one of three visceral nerves (N. cervicalis N. intestalis or N. analis).

(iv) Branching pattern of the GSN using electrophysiological techniques.

The data obtained in (i)-(iii) above was supplemented and confirmed using electrophysiological techniques. A suction electrode was used to record from or to stimulate the cut ends of the nerves emanating from the various ganglia. With an intracellular recording electrode in the GSN, orthodromic action potentials were recorded in the following nerves : Nervus pedalis superior, in the pedal ganglion; Nervus pallialis, in the right parietal ganglion; Nervus cervicalis, Nervus intestalis and Nervus analis in the visceral ganglion. (Nomenclature adapted from Bullock and Horridge (1965), and also from Elo (1938).

The extracellular spikes recorded in the five nerves named above followed the intracellular action potential in a one to one ratio, with constant latency even at high frequencies (10-20 spikes s^{-1}) (see for example Figure 24B-B'). These orthodromic action potentials also followed the intracellular spike in both high or low calcium media, conditions which would eliminate polysynaptic activity (Berry and Pentreath, 1976).

Stimulation of the cut nerve endings in which orthodromic spikes had been recorded, resulted in antidromic action potentials being observed in the GSN. These spikes did not change the amplitude, duration or latency with repetitive stimulation and disappeared when the soma was hyperpolarised (Figure 24 A-A^1). The antidromic action potentials also remained unchanged in the high calcium and calcium free media.

Figure 20.

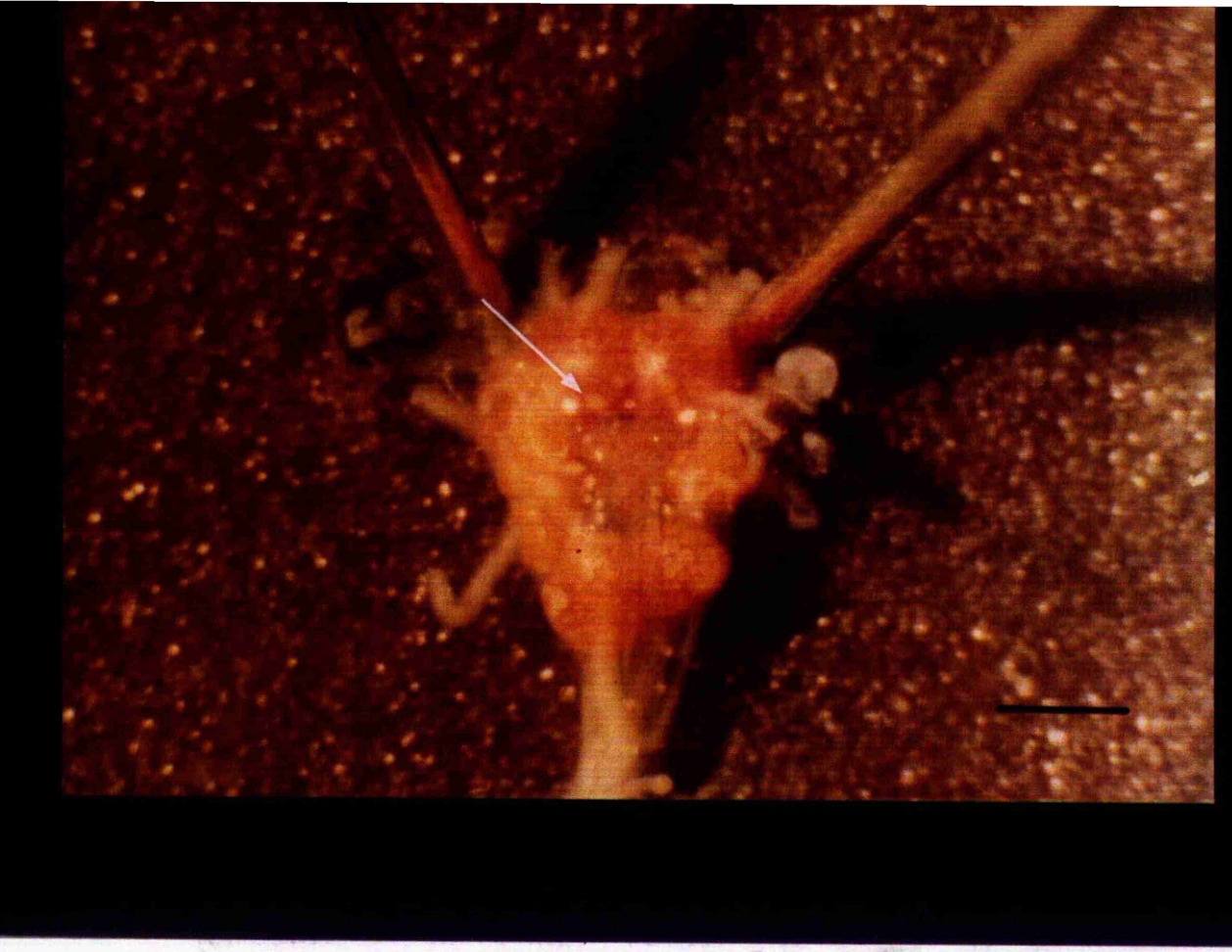
A. Whole mount photomicrograph of the CNS of Planorbis corneus : The serotonin neurone in the right pedal ganglion is arrowed. The large neurone in the opposite ganglion is the giant dopamine neurone (GDN, Berry and Cottrell, 1974). The intense white spots below each pedal ganglion are statocysts. Viewed from ventral surface.

Scale bar = 1 mm

B. Diagramatic representation of Planorbis CNS.

Key :

giant serotonin neurone,	GSN
giant dopamine cell,	GDC
left pedal serotonin neurone	LPSN
left/right pedal ganglion	L/RPG
left/right pleural ganglion	L/RPlG
left/right cerebral ganglion	L/RCG
left/right parietal ganglion	L/RPaG
visceral ganglion	VG



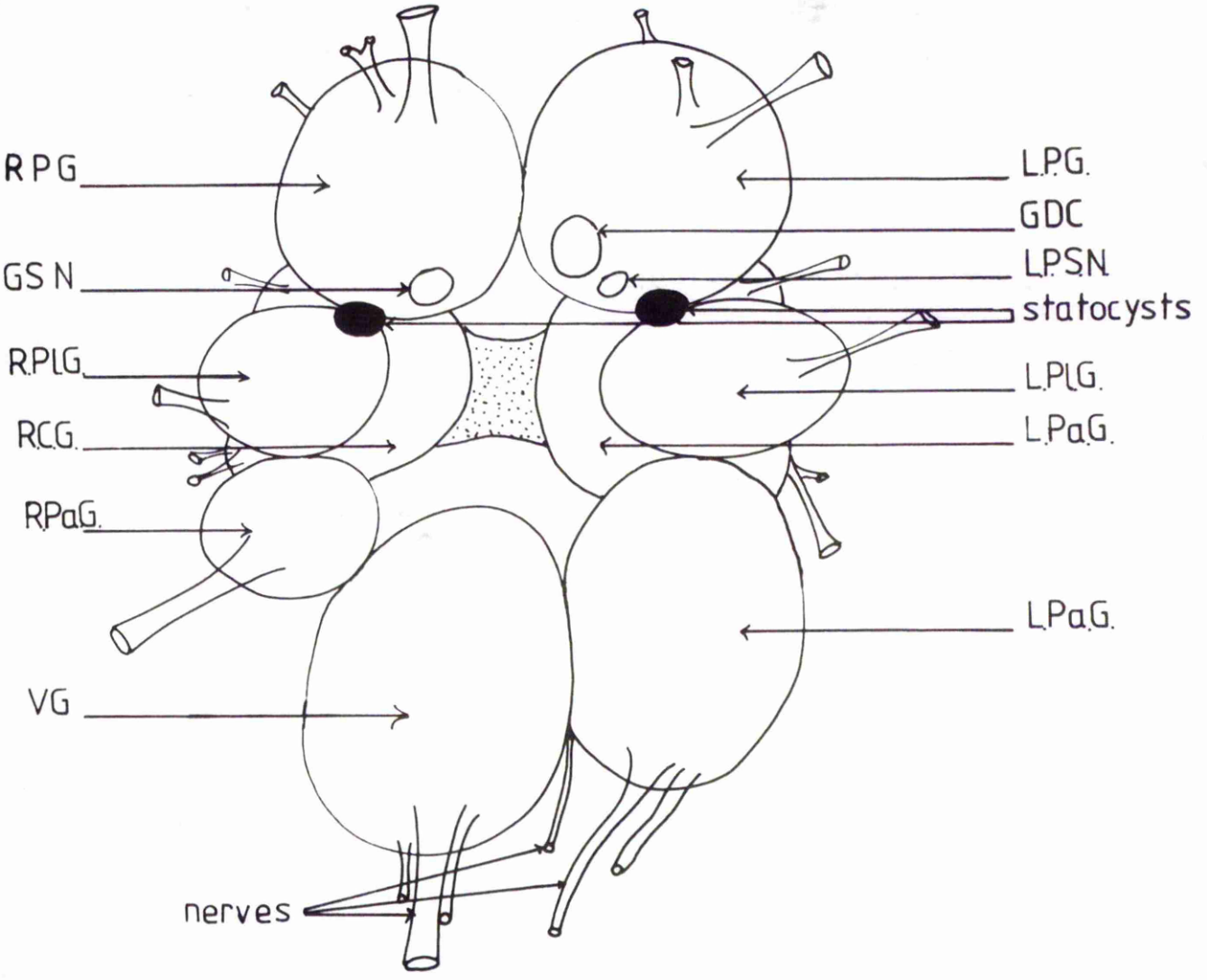

RPG
GSN
RPLG
RCG
RPaG
VG
nerves
LPG
GDC
LPSN
statocysts
LPLG
LPaG
LPaG

Figure 21.

Montage of photomicrographs from a whole mount of the CNS of Planorbis corneus after $CoCl_2$ injection into the right pedal ganglion serotonin neurone, viewed from the ventral surface.

Large open arrows show the main axon passing out of the pedal ganglion, travelling through the right pleural ganglion and into the right parietal ganglion. Large solid arrow indicates a major axon branch close to the cell body and small arrows show fine branches in pedal and pleural/parietal ganglia.

Scale bar = 150 µm.

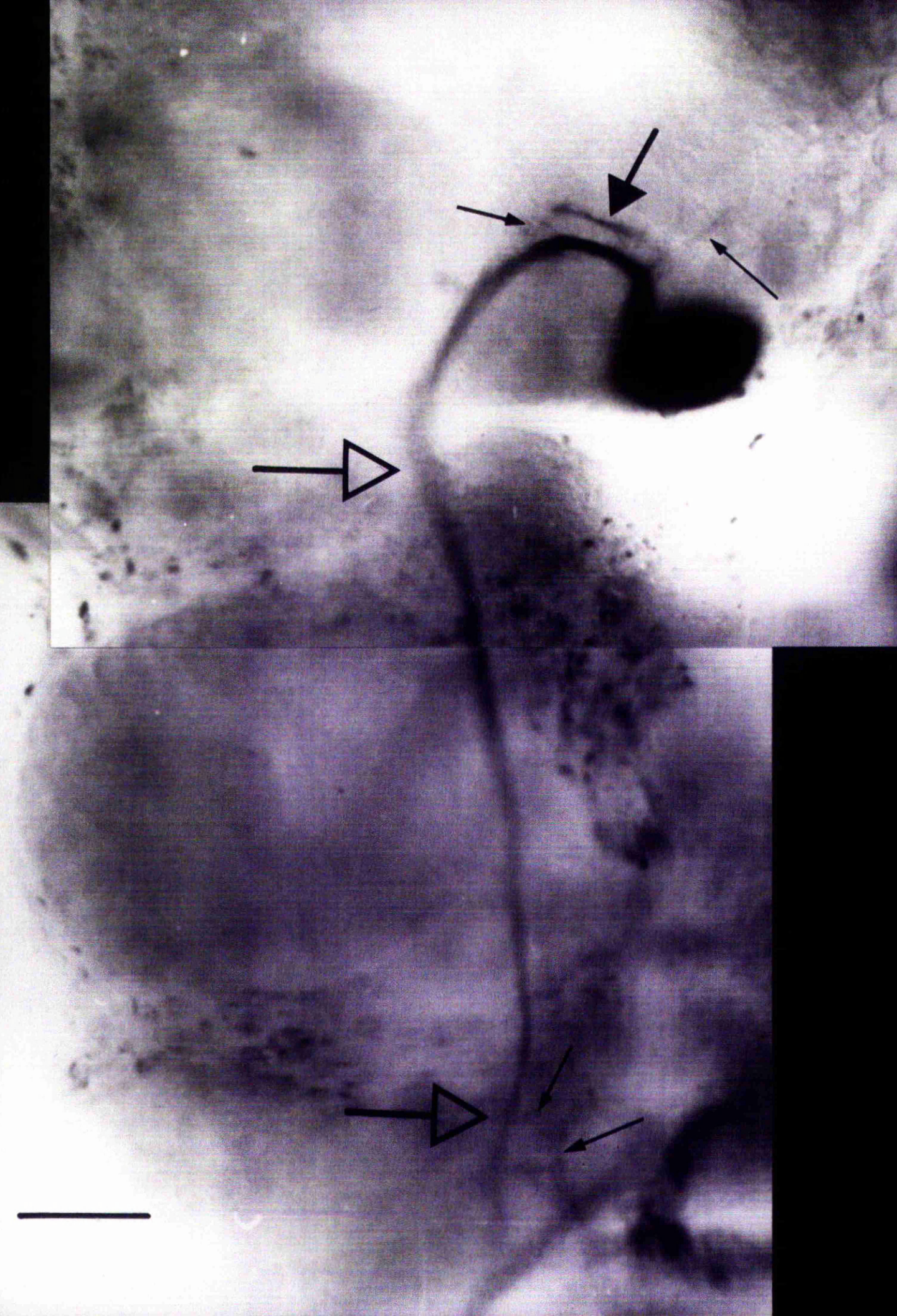


Figure 22.

Fluorescence photomicrographs of sections of the CNS of Planorbis corneus after Procion yellow injection into the right pedal ganglion serotonin neurone.

a) Section through soma of GSN showing large nucleus (n) and proximal segment of axon (arrowed).

b) Section through neuropil showing convoluted axon (large arrows) and fine branches (small arrow). Other neuronal somata (s) are clearly visible in this section.

c) Different neurone from a) and b) showing less convoluted axon than in b) (large arrows). Fine branches visible some distance from the main axon (small arrows), in the neuropil (N).

d) Same neurone as a) and b) showing extensive fine ramifications (small arrows) and one branch heading towards the contralateral pedal ganglion (uppermost small arrow).

Scale bar = 100 µm.

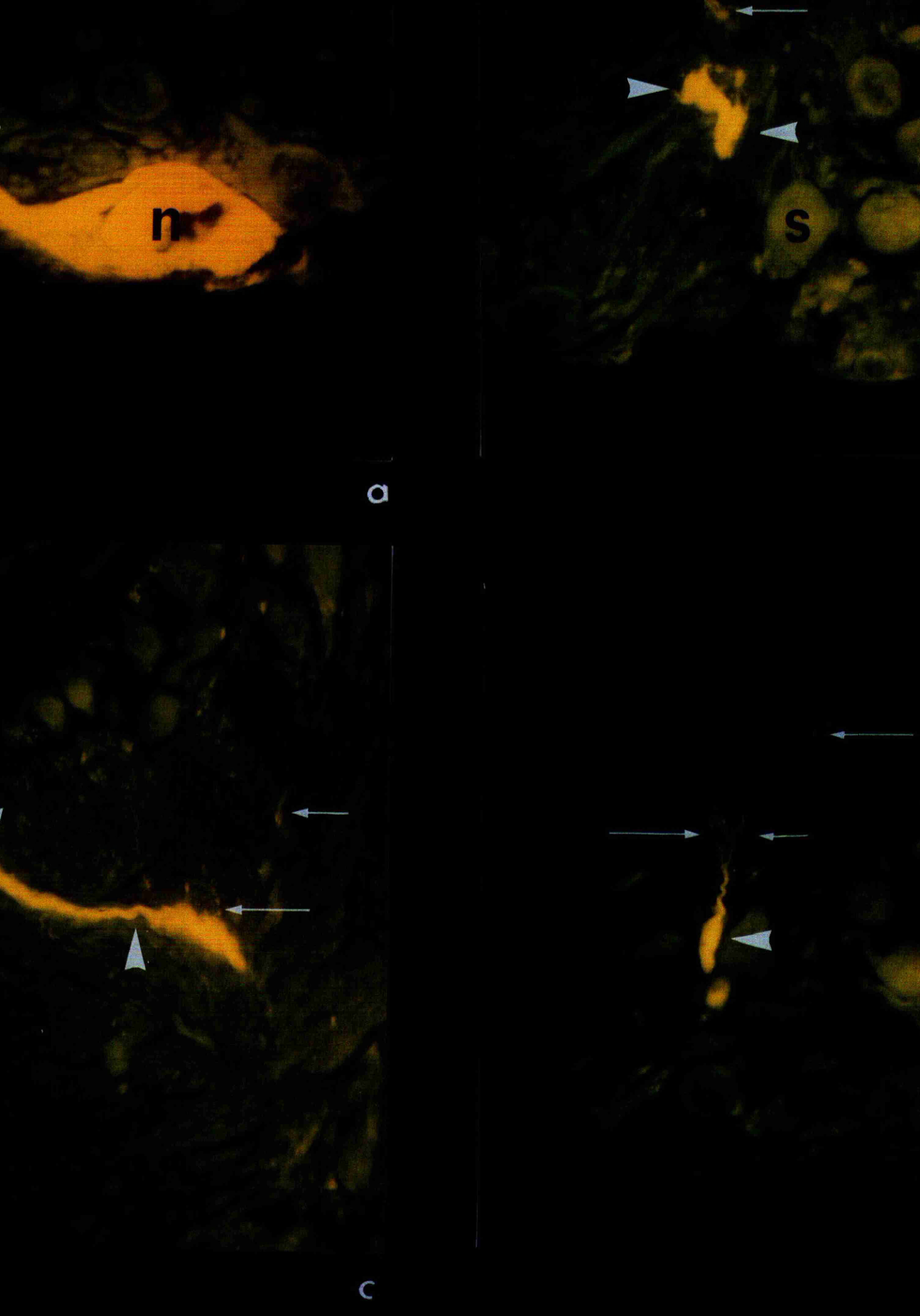

n
s
a
c

Figure 23.

Photomicrographs of autoradiograms of sections of the CNS of Planorbis corneus following intracellular injection of ^{3}H-5-HT into the giant serotonin neurone of the right pedal ganglion in three animals.

Dark field illumination and viewed from dorsal surface.

A. Section includes part of cell body (large arrow) main axon (double arrow), fine branches (small arrows) in neuropil (N) and in the direction of a pedal nerve (star).

B. Main axon of GSN passing out of pedal ganglion (large arrow). Small arrows show some fine collateral branches arborising from the main axon. Part of the cell body is also visible within section (solid arrow head).

C. Similar section to B above showing main axon on another neurone with fine branches projecting into the ganglion (small arrows). Double arrow indicates an axon branch in a nerve (probably a visceral or parietal nerve).

Scale bar = 100 µm.

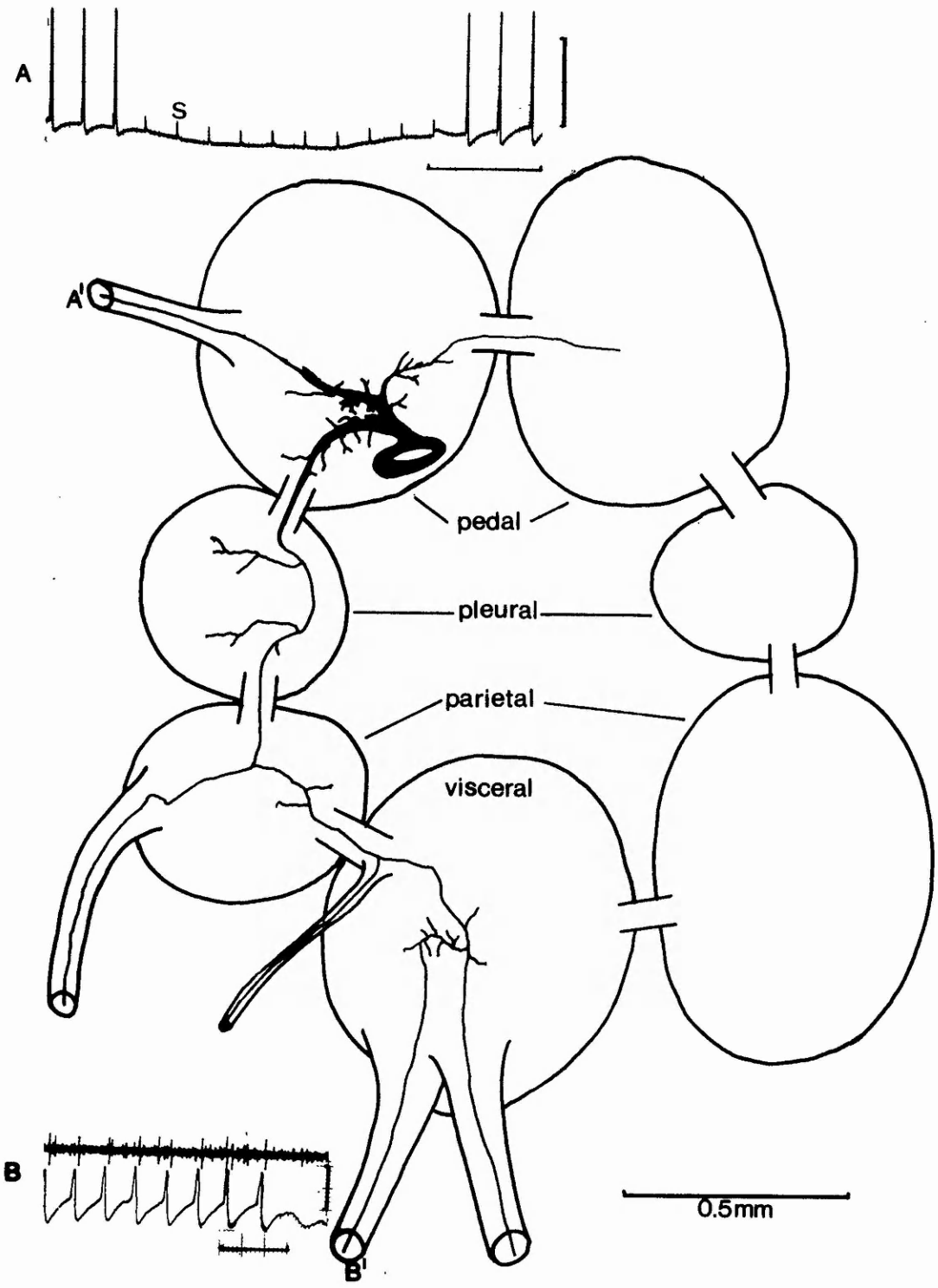

Z

Figure 24.

Diagramatic reconstruction of gross morphology of the giant serotonin neurone is the right pedal garglion of Planorbis.

This figure also includes representative electrophysiological data from studies used to examine nerve trunks for axons of the GSN.
A-A' Antidromic action potential (A) recorded in the GSN soma following stimulation of one pedal nerve (A'). The antidromic potentials are abolished as the neurone is artificially hyperpolarised and return as the membrane potential is returned to normal resting level. Only stimulus artifacts are visible in the hyperpolarised neurone (S).
B-B' Extracellular orthodromic action potentials recorded in visceral nerve B' (upper record) corresponding to intracellular action potentials recorded in GSN soma (lower record) at high frequencies (14 spikes. s^{-1})

Scales A 50mV, 30s
B 50mV, 150ms


A
S
A'
pedal
pleural
parietal
visceral
B
B'
0.5mm

5.3. (2) Synaptic connections of the GSN.

The giant-dopamine containing neurone GDN situated in an almost bilaterally symetrical position to the GSN, makes mono-synaptic connections with neurones in the visceral and parietal ganglia (Berry and Cottrell, 1975). Figure 25 demonstrates the connections made by the GDN with three different follower neurones in the parietal (A and B) and visceral ganglia (C and D). Stimulation of the GDN elicits discrete epsp's in neurone A which summate when the GDN is stimulated at higher frequencies (A and B). Neurone C exhibited smoothly summating ipsp's to GDN stimulation which elicited biphasic post synaptic responses in neurone D.

The morphology of the GSN is strikingly similar to the GDN (Berry, Pentreath, Turner and Cottrell, 1974; Pentreath and Berry, 1977) thus it seemed likely that the GSN might also make synaptic connections with neurones in the visceral and parietal ganglia.

More than 40 neurones were examined on the ventral surface of the visceral and parietal ganglia and their positions marked on a cell map as shown in Figure 26a. Many of the larger neurones were easily identifiable from preparation to preparation but it was impossible to repeatedly identify the smaller neurones or individual neurones from groups of cells.

Figure 27b shows the response of a ventral parietal neurone (labelled B in Figure 26) to stimulation of the GSN. Neurone B is a "follower" neurone of the GDN identified by its characteristic double action potential which is probably due to the fact that it is one of a group of electrically coupled neurones (Berry and Cottrell, 1977). When the neurone is hyperpolarised by about -15 mV below its resting level a slight depolarisation can be seen after stimulation of the GSN.

Figure 25.

Responses of "follower" neurones to intracellular stimulation of the giant dopamine neurone (GDN) of Planorbis corneus.

Upper trace is the intracellular record from the follower neurones and lower trace the intracellular GDN record. A and B are from the same neurone, C and D are different neurones.

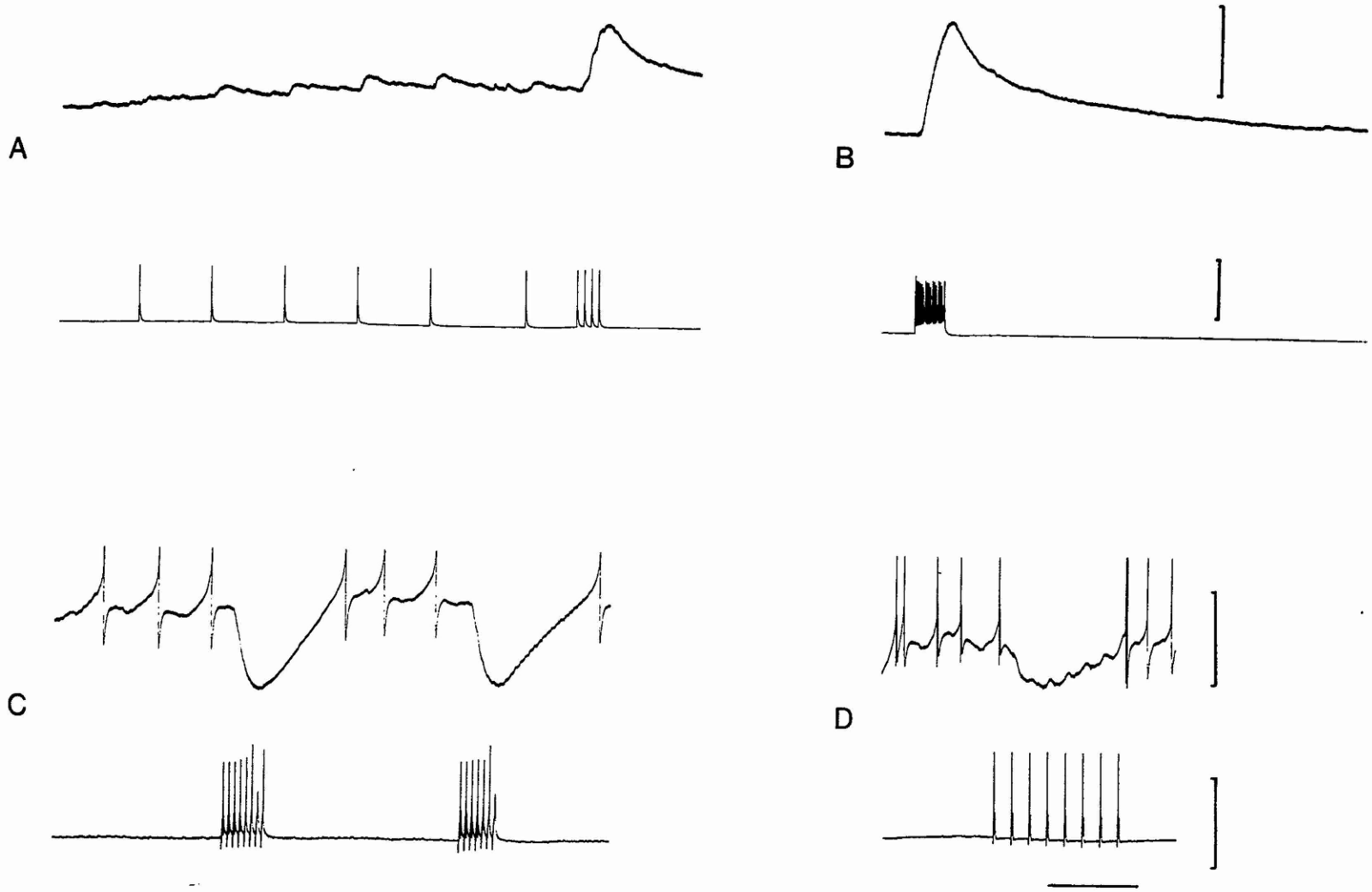
A. Discrete epsp's following GDN stimulation showing summation when four GDN spikes are produced consecutively.

B. Smooth summation of epsp's when a burst of action potentials is elicited in the GDN-

C. Neurone exhibiting smoothly summating ipsp's following GDN stimulation.

D. Smoothly summating ipsp's with discrete epsp's superimposed demonstrating biphasic iepsp's.

Scales Upper : 20mV
Lower : 100mV
Time : 5s


A
B
C
D

In B_1^- a second depolarising burst follows the first response and this can be repeated when the GSN is restimulated. The response appears to have a short latency in B_1, less than 2s, but in B_2 when the neurone is hyperpolarised by -5mV the latency appears to be 10-15s. The variability of the responses suggests that they are not monosynaptic in nature. The responses in this neurone should be compared to the results shown in Figure 25 where the responses of GDN follower neurones after GDN stimulation are shown. Stimulation of the GSN had no effect on the GDC elicited responses in this neurone.

No direct monosynaptic connections could be found in any of the visceral or parietal neurones when the GSN was stimulated. However stimulation of cell E. a small (less than 40 μm) unidentifiable neurone, situated near the edge of the visceral ganglion, elicited ipsps in the GSN. Hyperpolarisation of the GSN caused a reversal of the ipsp (see E_1, E_2). This neurone was found in only one experiment and consequently these results could not be repeated.

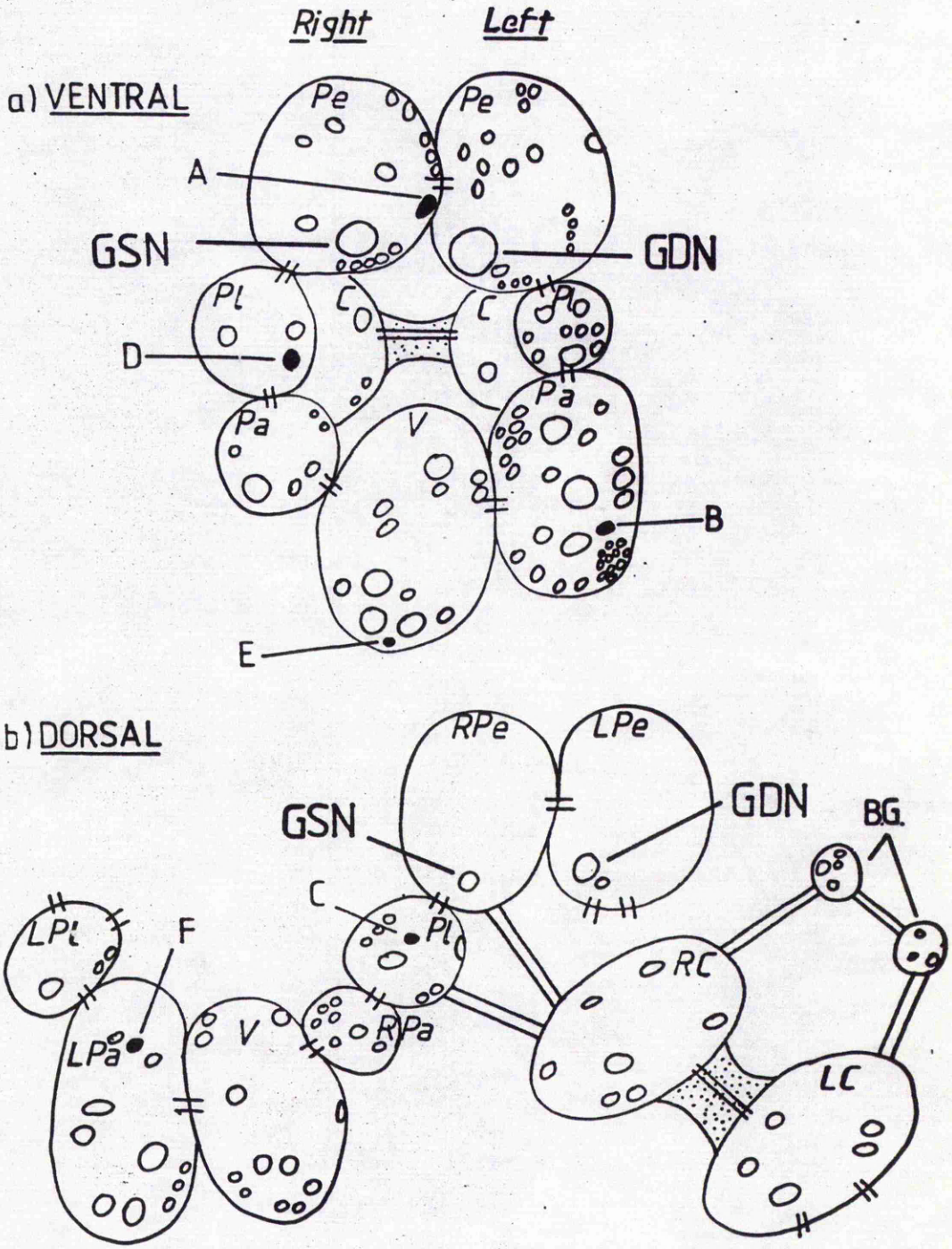
Figs 9A and D show records from two other ventral surface neurones. Neurone A was a large neurone (Ca. 100 μm) situated on the medial border of the right pedal ganglion, (see Figure 26a). Stimulation of the GSN elicited a depolarisation in neurone A at the resting level (A_1) and the response was repeatable and increased when the duration of the depolarising pulse was increased. When neurone A was hyperpolarised by -20mV stimulation of the GSN produced a slow depolarisation which lasted for some time after the stimulation ceased. No discrete epsp's could be seen and this response was abolished in high Ca^{++} solution suggesting a polysynaptic pathway may be involved.

Neurone D is located on the ventral surface of the right pleural

Figure 26.

Diagramatic map showing relative positions of identifiable neurones and unidentifiable groups of neurones in the CNS of Planorbis. Shaded circles and letters refer to neurones whose responses to GSN stimulation are shown in Figure 27.

Key : R, right; L. left
C, cerebral
Pa, parietal
Pe, pedal
Pl, pleural
V, visceral
BG, buccal ganglia


Right
Left
a) VENTRAL
Pe
Pe
A
GSN
GDN
Pl
C
C
Pl
D
Pa
Pa
V
B
E
b) DORSAL
RPe
LPe
GSN
GDN
B.G.
C
LPl
F
Pl
RC
LPa
V
RPa
LC

ganglion. This neurone exhibits spontaneous epsp's which can be clearly seen in this record when the neurone is hyperpolarised by -20mV. One burst of action potentials in the GSN produces a biphasic response in the follower neurone. This consists of a rapid epsp followed by a slower ipsp. This response appears spontaneously approximately 15s after the burst has ended suggesting that it is not due directly to the GSN but may be from an interneurone stimulated by the GSN and also stimulated as part of a reverberating circuit. The eipsp varied in latency and on three occasions a third response occurred about 30s after stimulation of the GSN.

At least 100 different neurones were examined on the ventral surface but no direct monosynaptic connections between the GSN and any other neurones were found. A similar procedure was carried out for the neurones on the dorsal surface and the cell map shown in Figure 26b demonstrates the position of the identifiable neurones.

Figure 9C shows the responses of a neurone on the dorsal surface of the right parietal ganglion. This neurone has a similar spontaneous activity to neurone D which was situated on the opposite surface of the same ganglion. Epsp's 10-20 mV in size can be easily seen when the neurone is hyperpolarised. Stimulation of the GSN reduces the size of the epsps to zero and when the GSN stops firing there is an inhibition in the neurone before the frequency of the epsp's returns to its previous level. A second burst of spikes in the GSN also reduces the size of the epsps and causes some inhibition. This effect was observed in several parietal and visceral neurones but great variation in the size of the responses made it impossible to quantify any of this data.

Neurone F also exhibits complex responses to stimulation of the GSN. This neurone in a rostromedial position on the dorsal surface of

Figure 27.

Responses of neurones in the CNS of Planorbis corneus to stimulation of the GSN.

Top trace "follower" neurones, bottom trace GSN.

A_1, Right pedal ganglion neurone exhibiting slight depolarisation on strong stimulation of the GSN.

A_2, Same neurone artificially hyperpolarised by -20mV exhibiting slight depolarisation (10mV; 50mV).

$B_{1,2}$ Parietal neurone, follower cell of GDN (see Figure 25D) exhibiting variable responses to GSN stimulation. (10mV, 50mV).

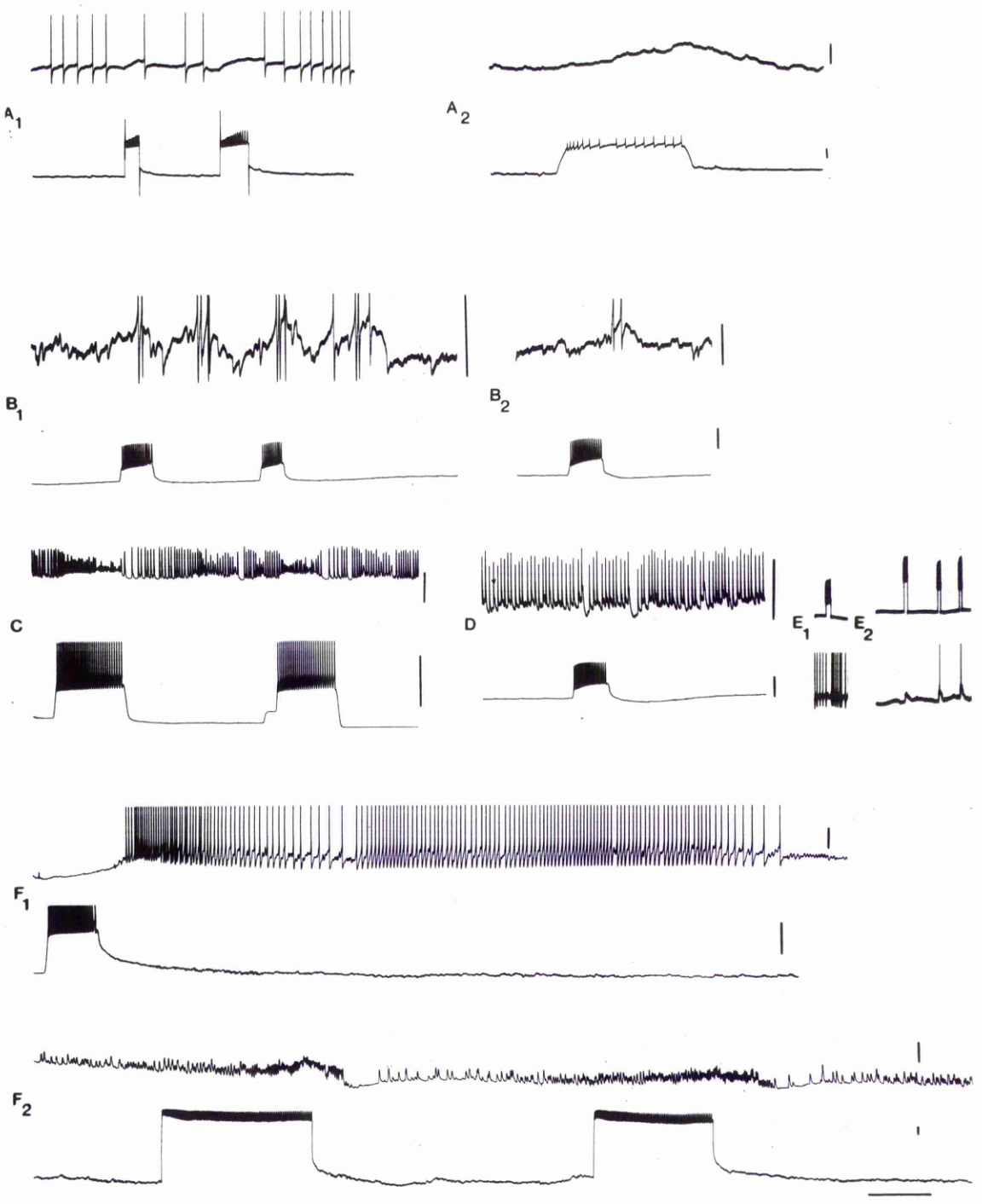
C. Pleural neurone hyperpolarised by -30mV below resting level (-50mV). Spontaneously occurring epsp's are reduced when the GSN is stimulated (10mV; 20mV).

D. Another pleural neurone also showing spontaneous epsps. GSN stimulation introduces a biphasic eipsp which reoccurs after stimulation ceases. (10mV; 50mV).

$E_{1,2}$ Small visceral neurone which produces an ipsp in the GSN when stimulated. E_2 shows reversal of the ipsps when the GSN is hyperpolarised by -25mV. (50mV, 20mV).

$F_{1,2}$ Parietal neurone exhibiting a complex response to GSN stimulation. The initial response consists of a short latency excitation followed by a longer latency excitation. When neurone F is hyperpolarised by -30mV before its resting level the complex effects of GSN stimulation on the spontaneous post-synaptic potentials can be seen (F_2). (10mV, 50mV).

Time scale = 30s
(5s for A_2).


A1
A2
B1
B2
C
D
E1
E2
F1
F2

the visceral ganglion was silent at its resting level. Stimulation of the GSN produced a depolarisation in neurone F resulting in a burst of spikes which became less frequent after 30s and several individual excitatory and inhibitory psp's were visible (9F). Approximately two minutes after the stimulation of the GSN was stopped a second increase in the activity of neurone F was seen.

This second response lasted for up to four minutes before the firing stopped. Discrete epsp's were also visible at the end of the burst of activity. When neurone F was hyperpolarised by 15mV below resting level (F_2) spontaneous epsp's could be seen. Stimulation of the GSN resulted in an increase in frequency but decrease in the duration of the individual epsp's. A slight wave of depolarisation can also be seen. Approximately 15s after the stimulation had ceased there was a total inhibition of the epsp's which lasted for 15s. This response could be repeated and almost identical effects were elicited 2.5 mins after the end of the first stimulus.

In the experiments designed to see if GSN stimulation had any effect on the responses of known follower cells to GDN stimulation no effects were observed. Figure 9B_1 and B_2 show responses of a GDN follower neurone (see above) and stimulation of the GSN before, during and after GDC stimulation had no apparent effect on the postsynaptic potentials produced by GDC stimulation.

More than 170 neurones were tested in all regions of the brain and no monosynaptic responses could be recorded. Most of the larger neurones were examined at least twice and many neurones were examined four or five times.

Between 30 and 40 neurones responded to stimulation of the GSN

but these responses appeared to be polysynaptic as they were inconsistant in form, duration and latency and in some experiments could be abolished by high calcium saline or calcium free, high magnesium saline.

5.3. (3) Iontophoresis experiments

Six putative transmitters were applied to the GSN and the intracellulary recorded responses observed. The results were taken from 17 different animals and each response was observed at least three times.

Iontophoretic application of ACh (10 nA, 100 ms) depolarised the GSN by about 5mV at the resting level. When the neurone was artificially hyperpolarised by about 25mV below the resting potential the depolarising response to ACh was more clearly seen; at this level of hyperpolarisation it was approximately 20mV and lasted 50s. (see Figure 28). After 30 minutes in a solution containing 10^{-3}M curare the response to ACh was abolished as was much of the activity elicited in the GSN by nerve stimulation. Dopamine and glutamate (both 10nA, 1.5s) hyperpolarised the neurone for 40-50s. The dopamine response was easily desensitised and a response could be elicited by switching off the retaining current rather than ejecting DA from the pipette. The responses to dopamine and glutamate were unaffected by 10^{-3}M curare whereas ergometrine 10^{-3}M totally abolished the dopamine response leaving the glutamate induced hyperpolarisation unchanged.

The GSN was unaffected by iontophoretic application of histamine, GABA and 5-HT (Figure 28). All of these substances were ejected with currents up to 200 nA for 2s but no responses could be observed even when the neurone was depolarised or hyperpolarised artificially. When histamine and GABA were applied to the GSN from a wide tipped pipette

(see 5.2.) in four out of five cases (histamine) and two out of four cases (GABA) hyperpolarisations were observed (Figure 6,Hp,Gp). No responses were elicited on other occasions. The hyperpolarisations (2-5mV) were slower in onset than the iontophoretic responses to dopamine and glutamate and lasted for up to one minute. 5-HT (Sp, Figure 28) elicited a bursting type activity in the GSN. Bursts of action potentials 2-12s in duration consisting of 5-20 spikes per burst lasted for up to six minutes after application of 10^{-2} 5-HT from a pipette. The activity was slow in onset (up to 30s) and was observed in four out of four experiments. A similar response could be evoked in the GSN following stimulation of a right pedal ganglion nerve.

Figure 28.

Responses of the GSN to putative transmitters.

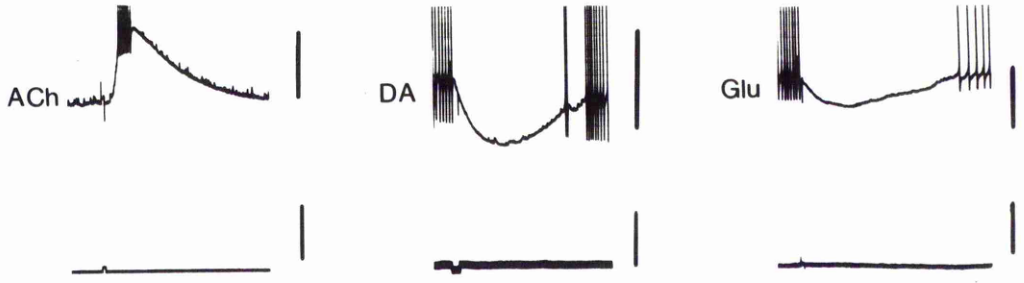
The top traces of the pairs of records show the intra somatically recorded responses of the GSN to iontophoretically applied transmitters. The lower trace is the iontophoretic current measurement. * (Scale bar = 50nA).

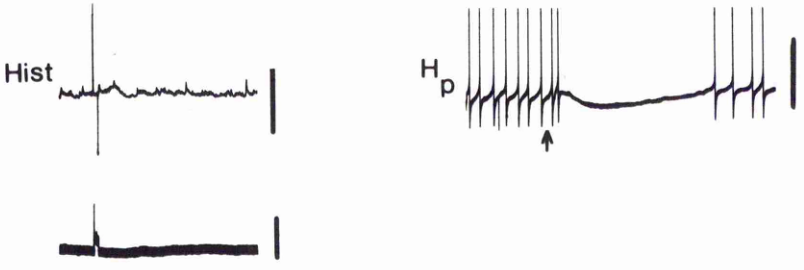
The responses to dopamine (DA) and glutamate (Glu) were obtained at the resting level whereas the responses shown to acetylcholine (ACh), histamine (Hist), γ amino-butyric acid (GABA) and 5-HT are at -15mV below the resting level.

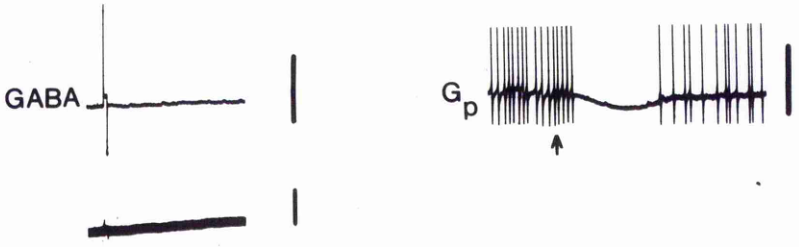
Hp, Gp and Sp show the responses of the GSN when 10^{-2}M histamine, GABA and 5-HT, respectively were applied to the soma from a wide tipped pipette. All three responses were recorded at the resting membrane potential.

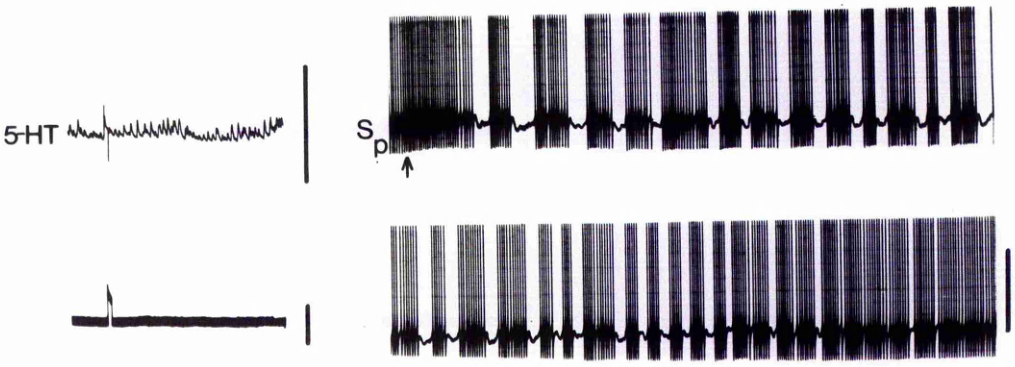
Scales 20mV, 50nA, 30s.

* inverted for DA response.


ACh
DA
Glu


Hist
Hp


GABA
Gp


5-HT
Sp

5.4. Discussion

Several giant molluscan neurones in the CNS of many species have been identified on the basis of their localisation, neuronal architecture, neurochemical and physiological properties (for examples, see books by Kandel, (1977) and Osborne (1977)). Such characterisation of these identifiable neurones has often facilitated studies on chemical transmission or the roles of identifiable neurones in observable behaviours.

This study was made to determine whether the serotonin-containing neurone in the right pedal ganglion of Planorbis corneus made monosynaptic connections with other neurones in the CNS. In order to facilitate the neurophysiological studies the morphology of the GSN was determined (to find regions containing GSN axons and terminals) and the GSN was characterised by its responses to putative transmitters (to aid visual identification). The first two sections of this discussion deal with the neuroanatomical properties (5.4. (1)) and the neuropharmacological properties (5.4. (2)).

Although almost one year was spent on this project and over 500 penetrations of potential follower cells were made no direct monosynaptic connections between the GSN and other neurones were observed. The final section of this discussion describes the results obtained with reference to more recent publications by other workers and discusses the significance of the recent data.

5.4. (1) Neuroanatomical studies

Intracellular injection of cobalt, Procion yellow, ^{3}H-5HT and ^{3}H-5HTP into the GSN permitted visualisation of the major axon branches and many finer branches at the light microscope level.

Cobalt injection was relatively successful when the tissue was viewed as a whole mount preparation. However only the soma, approximately 1 mm of the main axon and one major branch were clearly visible (see Figure 21). A few fine branches were seen arborising from the first major branch but smaller branches, which were numerous when other stains are used were not visualised. Although variation from preparation to preparation exists, especially in the numbers and distribution of finer dendrites it seems that cobalt is not a particularly effective stain when used in the pulmonate molluscs. For example Berry et al., (1974, and personal communication) found that cobalt filled only the first 1.5 mm of the giant dopamine-containing neurone axon of Planorbis and that small branches remained unstained. Similarly in two other pulmonates, Helisoma and Linea (Kaneko and Kater, 1973; and own observations with J.D. Turner) and in the opisthobranch Pleurobranchaea (Gillette and Davis, 1977) injected cobalt failed to stain both fine processes or to travel long distances down the axon in identified neurones. In comparison, $CoCl_2$ injection into insect motoneurones (Pitman et al.,1972) and central neurones in the opisthobranch Aplysia (Winlow and Kandel, 1977) has permitted visualisation of the neuronal architecture including many processes less than 1 µm in diameter. The reasons for the differences between species is unclear and may reflect differences in injecting technique (ie. pressure or current) or in the clearing agents used (eg. methyl salicylate or methyl benzoate). Procion yellow has been used previously to study identifiable neurones, neurosecretory cells and physiologically characterised neurones in several invertebrates (Stretton and Kravitz, 1968, 1973; Kerkut, French and Walker, 1970; Kater, Heyer and Hegmann, 1971; Sakharov and Salanki, 1971; Gillette and Pomeranz, 1973; Kaneko

and Kater, 1973; Cottrell and Macon, 1974; Blackshaw, 1976). Previous studies (Pitman et al., 1972; Kaneko and Kater, 1973) have suggested that Procion yellow dyes will not stain fine processes in the cockroach or in some gastropod molluscs. However in this study and in the lobster (Stretton and Kravitz, 1973) and in some vertebrates, fine dendrites and dendritic spines have been filled with the dye (Jankowska and Lundstrom, 1973; Llinas, 1973; and see Kater and Nicholson, 1973).

The introduction of a radioactively-labelled substance and its subsequent visualisation by autoradiography has also been widely used to study neuronal geometry and synaptic connections. This technique, introduced by Globus, Lux and Schubert, (1968) has been applied in molluscs to locate axonal pathways and synaptic terminals of characterised neurones (Pentreath et al., 1974; Pentreath and Cottrell, 1974; Pentreath, 1976; Thompson et al.,1976 Pentreath and Berry, 1977). In this study radioisotope injection followed by autoradiography provided the most detailed description of the fine branching pattern of the GSN. The extensive dendritic tree which was not seen using cobalt as a stain was clearly visible in the autoradiograms (see Figure 23).

Although electrophysiological methods can give no information about the dendritic tree and fine branching patterns of molluscan neurones, such methods have been used extensively to indicate the presence or absence of axon branches in particular nerves (Tauc and Hughes, 1962; Kandel and Tauc, 1966a; Dorsett, 1967; Berry and Pentreath, 1976a; Blackshaw, 1976; Gillette and Davis, 1977) The orthodromic and antidromically evoked responses shown in Figure 24 confirmed the

presence of GSN axon branches in five nerves. In each preparation the branches in pedal, intestinal and anal nerves were invariant but the other two branches exhibited a considerable variability from preparation to preparation. This may have been due to an inability to record action potentials from fine axon branches or possibly to the complete absence of a branch in these nerves. The variability of axon branches in particular nerves has been reported previously in Aplysia following $CoCl_2$ injection (Winlow and Kandel, 1976) and its significance is not clearly understood.

It was difficult to quantify exactly the extent and number of the fine branches of the GSN by any of the intracellular stains used as there was a considerable variation from preparation to preparation. Nevertheless, the main axon and the major branches were constantly observed within the ganglion ring in each experiment. As the primary aim of using these techniques was to identify the major axonal pathways with a view to locating the terminals of the GSN, a quantitative analysis of the fine branching pattern was not attempted. Hence the diagramatic representation of the dendritic tree in Figure 24.

In conclusion, all three staining techniques plus the electrophysiological data provided information about the neuronal geometry of the GSN and helped identify the regions most likely to contain terminals of the neurone thus indicating areas most likely to contain neurones which might receive monosynaptic inputs from the GSN.

5.4. (2) Amine and amino acid receptors on the soma of the Planorbis serotonin neurone.

The somata of gastropod neurones, although devoid of synapses, contain receptors to many low molecular weight compounds (see review by Gerschenfeld, 1973, Ascher and Kehoe, 1975; Kehoe and

Marder, 1976). The GSN responded to localised iontophoretic application of three out of six putative transmitters in invertebrates and to perfused application of the remaining three.

ACh is probably an excitatory transmitter acting on the GSN as curare blocked both the iontophoretic response and the excitatory responses evoked by nerve stimulation. Curare has also been shown to block excitatory dopamine (Ascher, 1972) and 5-HT responses (Cottrell and Macon, 1974) but neither of these amines produced excitations when applied iontophoretically. Iontophoretic dopamine and glutamate both hyperpolarised the GSN and both substances have been postulated as inhibitory neurotransmitters in the snail (Szczepaniak and Cottrell, 1973; Berry and Cottrell, 1974). Interestingly, glutamate is very likely an inhibitory transmitter acting on the serotonergic metacerebral neurone of Helix (Szczepaniak and Cottrell, 1973). It was impossible to distinguish whether dopamine or glutamate (or both?) were inhibitory transmitters acting at physiological synapses on the GSN since nerve stimulation generally produced powerful excitations in the neurone and isolated inhibitions were rarely seen.

The responses to histamine, and 5-HT indicate that the GSN probably does not receive a monosynaptic input mediated by either of these putative transmitters. It is conceivable however, that the receptors to these compounds are selectively localised on the axon or dendrites of the GSN are therefore not freely accessible to iontophoretically applied substances. Dopamine receptors in Aplysia (Ascher, 1972) and different acetylcholine receptors in Navanax (Levitan and Tauc, 1972) have been demonstrated on the axons but not the superficial borders of the somata of identifiable neurones. This may explain the slight differences in responses observed in this study and an earlier one

which used bath application of drugs (Loker, Walker and Kerkut, 1975). Differences in methods of application of drugs have produced different responses in previous studies in Planorbis (Logan and Cottrell, 1974).

5.4. (3) Synaptic connections made by the serotonin neurone.

Stimulation of the 3SN evoked responses in many neurones in the CNS of Planorbis. All the responses recorded were polysynaptically evoked since they were inconsistant and abolished in calcium free or high calcium media (Berry and Pentreath, 1976b). In comparison, the responses evoked by stimulation of the giant dopamine neurone (Figure 25) are clearly monosynaptic (see Berry and Cottrell, 1974). It is conceivable that the majority of neuronal interconnections in molluscs are made in an indirect manner. The complex neuronal geometry of the neuropil might facilitate such an occurrance since pre- and postsynaptic sites from many neurones in close apposition in this region.

The absence of axo-somatic synapses in molluscs (Coggleshall, 1967) means that electrophysiological recordings made in the soma of a molluscan neurone reflect electronically conducted potentials which have arisen in the dendrites at some distance from the soma. The difference in cable constants and resistances between the axon and the soma (see Katz, Chapter 6, 1966) may mean that electrical events at the dendrites may not be transmitted to the soma. Antidromic spikes in Aplysia often fail to invade the soma of identified neurones although conduction between different branches of the axon is normal (Tauc and Hughes, 1962). Thus it is possible that monosynaptic connections could exist between neurones although they are not observed by intrasomatically situated electrodes.

Data published from studies made since these experiments

were carried out have demonstrated that iontophoretically applied 5-HT can evoke a response in Aplysia neurones, one component of which may be due to the activation of a voltage-sensitive conductance change. (Pellmar and Wilson, 1977). This contrasts with the previously held view that conventional synaptic or iontophoretic responses to 5-HT result from voltage independent conductance changes. There is now a substantial body of evidence which suggests that 5-HT is a mediator of hetero-synaptic facilitation in Aplysia (Kandel, Brunelli, Byrne and Castelluci, 1975; Shimahara & Tauc, 1975; Tremblay et al., 1976; Woodson et al., 1976). 5-HT exerts these effects by increasing the synthesis of cAMP which in turn causes an increase in the active permeability to calcium at the neurone terminals thus facilitating transmitter release (Shimahara and Tauc, 1977; Klein and Kandel, 1978; Weiss et al.,1978).

Furthermore, Pellmar and coworkers (1977,1979) have demonstrated that some Aplysia neurones possess 5-HT receptors which activate a voltage-dependent calcium channel when stimulated by iontophoretically applied 5-HT. Moreover this 5-HT response could not be observed unless potentials above - 30 mV were sampled (Pellmar and Wilson, 1977) and since many molluscan neurones exhibit rectification (Kandel and Tauc, 1966; Gerschenfeld and Paupardin-Tritsch, 1974, and own unpublished observations with M.S. Berry in Planorbis) this response would not be observed by conventional recording techniques and voltage clamping would be required (Pellmar and Wilson, 1977).

Since voltage-clamp techniques were unavailable when these experiments were made in Planorbis it is interesting to speculate whether such unconventional 5-HT responses are mediated by the Planorbis serotonin neurone. The variable effects induced by GSN stimulation were considered to be polysynaptic if abolished in calcium free bathing

medium. However the evidence that 5-HT can mediate its effects via a calcium current (Pellmar & Wilson, 1977; Pellmar and Carpenter, 1979) means that removing calcium from the medium is an erroneous test of monosynaptic activity.

Furthermore since one role of 5-HT is to modulate presynaptic transmitter release (see above) then perhaps more attention should have been paid to the effects of GSN stimulation on evoked responses in other neurones. Such experiments were attempted and negative results were obtained on the effects of GSN stimulation on dopamine neurone evoked responses in GDN follower cells (see Results). A major problem with this type of experiment was that it was difficult to obtain a "pure" response in any particular neurone since nerve stimulation generally produced a mixture of excitatory and inhibitory responses. Thus it was impossible to determine the effects of GSN stimulation on these responses.

Nevertheless, the responses shown in Figure 27 clearly demonstrate that stimulation of the GSN did modulate the spontaneous activity in other neurones in the brain but whether these effects were as a direct result of a presynaptic action of 5-HT awaits more definative experiments.

In conclusion, although a great deal of time and effort was spent on this section of the thesis, the results were disappointingly negative. The Planorbis GSN does not appear to make monosynaptic connections with other identifiable neurones in the CNS and is therefore unlikely to provide a good model in which to study the role of 5-HT as a conventional neurotransmitter. Nevertheless, this system may provide an opportunity to examine the possibility that 5-HT acts as a neuromodulator in the molluscan CNS and in the light of recent

developments, a reappraisal of the methodology used suggest that more sophisticated techniques will be required to establish connections between the GSN and other neurones. In addition, since the GSN can affect the activity of many neurones in the CNS and since it is readily identifiable, in vivo studies in the future may help elucidate the function of this neurone in the behaviour of the animal.

Chapter 6 GENERAL CONCLUSIONS AND SUMMARY

"When we are dealing with two different endings of the same sensory neurone, the one peripheral and concerned with vasodilatation and the other at a central synapse, can we suppose that the discovery and identification of a chemical transmitter of axon-reflex vasodilatation would furnish a hint as to the nature of the transmission process at a central synapse?"

H.H. Dale, 1935

The above question posed more than forty years ago and subsequently modified by Eccles (see Eccles, 1976) to the concept that a neurone will release only one transmitter from all its endings has come to be known as Dale's Principle. A recent repraisal of the evidence for and against this Principle (Burnstock, 1976) has suggested that the concept of one neurone - one transmitter may not be universally applicable and that Dale's Principle should be re-examined in the light of many and more sensitive neurochemical techniques.

The development of such sensitive assay techniques has demonstrated that many molluscan neurones contain more than one putative transmitter (see Kehoe and Marder, 1976). Furthermore, an identifiable neurone in Helix may release both ACh and 5-HT at a physiological synapse (see Cottrell, 1977). Thus, in molluscs there is evidence that Dale's Principle may not apply to all neurones. The data reported in this study demonstrate that of all the neurones studied in the central ganglia of Helix aspersa, Helix pomatia and Planorbis corneus only the metacerebral neurones of Helix can synthesise both ACh and 5-HT from their precursors. Other aminergic or cholinergic neurones can synthesise either 5-HT or ACh respectively but not both transmitter substances.

Newly synthesised 5-HT is transported to the endings of the GSC

and released there. In addition to transporting and releasing 5-HT the GSC's can transport and possibly release newly synthesised ACh although the transport mechanism may be different from the mechanism of 5-HT transport.

This study also describes some of the properties of an identifiable 5-HT-containing neurone in Planorbis and reviews some recent literature which suggests that 5-HT acts as a neuromodulator in the molluscan CNS. Consequently 5-HT and ACh may subserve different functions if both are released from the GSC.

In conclusion, this study supports the idea that a neurone can synthesise more than one transmitter substance. The evidence that more than one transmitter is released is still circumstantial and will have to await a more sensitive and sophisticated method for the detection of released acetylcholine in molluscs.

Nevertheless, the electrophysiological data (see Cottrell, 1976, 1977) strongly suggests that two transmitters are involved. Moreover the morphological evidence of two vesicle types (Pentreath, 1976) and the biochemical studies (Emson and Fonnum, 1974; Hanley et al., 1974; this study) all indicate that there is at least circumstantial evidence that the Helix GSC s can synthesise, transport and release more than one transmitter substance. This likelihood makes it appropriate to conclude with the final sentence of Sir Henry Dale's quotation above .....

"The possibility has at least some value as a stimulus to further experiment."

SUMMARY

1. Studies have been made on identifiable neurones in the central nervous system of some gastropod molluscs.
2. Using a technique of intracellular injection of radiochemicals by pressure the transmitter synthesising capabilities of identifiable neurones have been investigated.
3. Methods have been developed to ensure that radioactive metabolites detected within the CNS following intracellular injection are within, and confined to, the soma, axons, dendrites and terminals of the injected neurones.
4. Identifiable 5-HT containing neurones, will synthesise ^{3}H-5-HT from injected ^{3}H-tryptophan and injected ^{3}H-5-HT. Newly synthesised ^{3}H-5-HT in the Helix pomatia metacerebral 5-HT containing neurones is transported to the terminals of the neurone and probably released there.
5. Identifiable cholinergic neurones in Helix pomatia can synthesise ^{3}H-ACh from ^{3}H-choline but not ^{3}H-5-HT from ^{3}H-tryptophan or ^{3}H-5-HTP.
6. Two aminergic neurones in Planorbis corneus, a dopamine containing neurone and a 5-HT containing neurone, can synthesise ^{3}H-5HT from its precursors but not ^{3}H-ACh from ^{3}H-choline.
7. The metacerebral 5-HT containing neurones of Helix pomatia and Helix aspersa alone possess the enzymes for both ^{3}H-ACh and ^{3}H-5-HT synthesis from their precursors.
8. In addition there is evidence that newly synthesised ^{3}H-ACh within the Helix pomatia GSC s can reach the neurone terminals in the buccal ganglion and can possibly be released there.

9. An extensive, neuroanatomical, neurochemical, neurophysiological and pharmacological study has been made of an identifiable 5-HT containing neurone in the right pedal ganglion of Planorbis corneus. There is no evidence that this neurone makes conventional monosynaptic connections with other neurones in the brain but the role of 5-HT as a presynaptic modulator is discussed.

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